UNIVERSITY OF ALBERTA

ISOLATION, GROWTH AND METABOLISM OF RHODOCOCCUS STRAIN S1 ON ANTHRACENE

by

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©

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TO MY PARENTS

For their unconditional love and encouragement

ABSTRACT

Three acid-fast bacteria were isolated from a mixed culture enriched for growth on anthracene, using creosote-contaminated soil as the inoculum. These three isolates could degrade anthracene as shown by the production of a clear zone around the colony after growth on a mineral salts agar plate sprayed with anthracene. All three isolates were identified as members of the genus *Rhodococcus*. Growth of the isolates was slow on crystalline anthracene with a doubling time of 1.5-3 days. All three isolates mineralized anthracene but not phenanthrene or naphthalene. Of 11 polycyclic aromatic hydrocarbons (PAHs) tested, only anthracene served as sole source of carbon and energy for growth but not naphthalene, biphenyl, acenaphthene, phenanthrene, fluorene, fluoranthene, pyrene, chrysene, or 1,2-benzanthracene. One isolate, *Rhodococcus* sp. strain S1, was able to use 2-methylanthracene or 2-chloroanthracene as carbon source but not 1- or 9-substituted analogs. These results suggest that the initial enzyme attacking anthracene in these isolates has a narrow substrate specificity.

The metabolism of anthracene by *Rhodococcus* sp. strain S1 was further investigated. To compare the established pathway for anthracene catabolism in Gram negative bacteria with that of *Rhodococcus* S1, *Pseudomonas fluorescens* LP6a (pLP6a :: *Tn*5) mutant D1, known to produce 1,2-dihydroxy-1,2-dihydroanthracene (anthracene 1,2-dihydrodiol), was used for comparative studies. Both organisms were grown on anthracene mineral salts medium. Underivatized and trimethylsilyl-derivatized extracts were analyzed by thin-layer chromatography, gas chromatography with flame ionization detector, gas chromatography-mass spectrometry, and gas chromatography-Fourier Transform infrared spectrometry. *P. fluorescens* LP6a mutant D1, when incubated with anthracene was shown to produce anthracene 1,2-dihydrodiol as the initial metabolite as reported in the proposed pathway. *Rhodococcus* S1, when grown on anthracene was

shown to cometabolically transform phenanthrene present as a 1% contaminant in the purest commercially available anthracene, to yield 9,10-dihydroxy-9,10-dihydrophenanthrene (phenanthrene 9,10-dihydrodiol). Comparison of this phenanthrene 9,10-dihydrodiol and its trimethylsilyl and butaneboronic acid derivatives with the chemically synthesized phenanthrene *trans*-9,10-dihydrodiol indicated that phenanthrene 9,10-dihydrodiol from *Rhodococcus* S1 was in the *trans* configuration. This transformation product and others obtained from a variety of PAHs were derived from cytochrome P450-like monooxygenase activity as shown by studies with the specific inhibitors metyrapone and ancymidol. Less phenanthrene 9,10-dihydrodiol and chrysene dihydrodiol were observed when metyrapone and ancymidol were present in the growth medium of anthracene-grown *Rhodococcus* S1 incubated with phenanthrene and chrysene, respectively. Of 11 PAHs tested, *Rhodococcus* S1 cometabolically transformed naphthalene, biphenyl, acenaphthene, phenanthrene, fluorene, fluoranthene, pyrene, and chrysene but not 1,2-benzanthracene, naphthacene, or 2-methylphenanthrene. Twenty one oxidized metabolites of these PAHs were identified by molecular ions and mass spectral fragmentation patterns, and in six cases by comparison of GC retention times with authentic standards.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BHI	Brain heart Infusion
BSA	*N,O*-bis(trimethylsilyl)acetamide
BTB	Butaneboronic acid
DCM	Dichloromethane
DMF	*N,N'*-Dimethylformamide
EDTA	Ethylenediamine tetra acetic acid
EtAc	Ethyl acetate
FID	Flame ionization detector
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
HP	Hewlett Packard
HPLC	High performance liquid chromatography
Km	Kanamycin
IARC	International Agency for Research on Cancer
NB	Nutrient broth
NMR	Nuclear magnetic resonance spectrometry
MS	Mass spectrometry
MW	Molecular weight
OD	Optical density
PAH	Polycyclic aromatic hydrocarbon
PCA	Plate count agar
SEM	Scanning electron microscopy
SDS	Sodium dodecyl sulfate

TLC	Thin layer chromatography
TMS	Trimethylsilyl
TSB	Trypticase soy broth

1. INTRODUCTION.

1.1 Bioremediation and history of the research.

1.1.1 Bioremediation.

The research in this thesis resulted from initial observations made in a collaborative project between the Departments of Chemical Engineering and Microbiology at the University of Alberta. The objective of this collaboration was to investigate the enhancement of bioremediation of creosote-contaminated soil using low aqueous phase soil slurries in a rotating drum bioreactor.

Bioremediation is a method to clean the creosote-contaminated soil in which microorganisms are employed in the process of destroying the contaminating organic compounds by converting them into carbon dioxide and biomass. This biological treatment technology depends on the degradative activities of microorganisms. It emphasizes enhancing existing but slow biodegradation processes in nature, or technologies that bring the contaminating chemicals into contact with microorganisms, in some type of reactor that allows for rapid transformation (Alexander 1994). The ultimate goal of bioremediation is to degrade organic pollutants to concentrations that are either undetectable or, if detectable, to concentrations below the limits established as safe or acceptable by regulatory agencies. Bioremediation has been used to degrade organic contaminants in soils, ground water, wastewater, sludges, industrial-waste systems, and gases. It is usually used as the last method after physical removal, when the contaminant level is 2% or less (Alexander 1994).

Bioremediation of soil polluted with hydrocarbon contaminants can be performed in various ways such as *in situ* treatment, land farming, heap treatment, and agitated tanks (Gray *et al.* 1994). Successful bioremediation depends on several factors. For example, oxygen and nutrients must be provided sufficiently to enable rapid conversion of organic

contaminants by either indigenous or inoculated microorganisms. The contact between the active microorganisms and the contaminated soil needs to be provided efficiently to ensure complete removal of organic pollutants and avoid leaving secluded zones untreated. Land farming has been frequently used by the oil industry to destroy oily wastes or the hydrocarbon-rich materials that are spilled on soil. The method relies on activities of the indigenous microorganisms, and nitrogen and phosphorus are often added to the contaminated soil, normally in the form of commercial fertilizers, to support a large biomass (Alexander 1994). This method has the advantage of the simplicity of the process and low costs. However, one limitation of this method is the lack of control. Excavation of contaminated soil for controlled treatment is of interest in cases of surface contamination, in soil with poor hydraulic conductivity, and in cold climates where rates of biological conversion *in situ* would be very low. Organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) have a very low water solubility resulting in their bioconversion being controlled by mass transfer. In this case, a soil-slurry bioreactor could be chosen for soil bioremediation because it enhances the conversion rates of insoluble PAHs (Gray *et al.* 1994).

1.1.2 Biological treatment of creosote-contaminated soil using a rotating-drum bioreactor.

The collaborative research team had developed a biological soil cleaning technique using an anthracene-utilizing mixed bacterial culture together in a rotating drum bioreactor (Gray *et al.* 1994, Banerjee *et al.* 1995). Soil impregnated with anthracene was used as a model for bioremediation of soils contaminated with PAHs. Anthracene was chosen as a model compound for the following reasons: (i) anthracene is a three fused benzene ring compound which belongs to a group of polycyclic aromatic hydrocarbons (PAHs) that accounts for about 85% of the creosote component (Mueller *et al.* 1989) (ii) it has

potentially mutagenic and carcinogenic effects (IARC 1983) and (iii) its water solubility is very low (45 µg/L, May *et al.* 1978) which makes it very difficult for bacteria to utilize this compound. If anthracene could be completely degraded it was conceivable that other creosote fractions that had higher water solubility would also be readily degraded.

Among the various types of bioreactors, the slurry-phase, rotating-drum bioreactor was employed because it provided effective mixing with high solids content and high aeration rates, and it is prefered over the *in situ* treatment for contaminated surface soils (Banerjee *et al.* 1995). The soil was prepared as a 60% weight slurry in salts medium and inoculated with a mixed bacterial culture that had been selected on the basis of its ability to utilize anthracene as the sole carbon and energy source for growth. The use of an active bacterial mixed culture in the rotating-drum bioreactor resulted in rapid degradation of anthracene in repeated batch operations. The bacterial population remained active over the 120-day test period with up to 80% of the reactor contents being replaced with freshly contaminated soil (Banerjee *et al.* 1995).

1.1.3 Examination of the anthracene-utilizing, mixed bacterial culture.

The mixed bacterial population was derived from the indigenous bacteria present in creosote-contaminated soil. This population was selected based on its ability to utilize anthracene as the sole source of carbon and energy for growth and was maintained by monthly transfers of 10% (v/v) of settled culture supernatant to fresh mineral salts medium containing anthracene. Examination of the mixed bacterial population in the maintenance culture after the sixth monthly transfer by plating on plate count agar revealed two populations differentiated by their growth rates: the fast and slow growing populations (Gray *et al.* 1994). The fast growing population appeared as large colonies on plate count agar plates after two days of incubation and were identified as Gram negative

Pseudomonas spp. The slow growing population appeared as small colonies on PCA plates after one week of incubation. They were Gram positive and stained acid-fast (Gray *et al.* 1994). Since these bacteria were acid-fast, they were members of the actinomycetes. The slow growing, anthracene-utlizing population showed their ability to degrade anthracene by the appearance of clear zones around colonies streaked on mineral salts agar plates and sprayed with a solution of anthracene in dichoromethane (Tongpim and Pickard 1996). No fungal colonies were detected on PCA or anthracene-mineral salts agar plates.

It was found that the slow growing population always existed and appeared even in the situation where they were numerically dominated by the fast growing population. More over, at the time of starting this work, several reports describing microbial degradation of anthracene had been published (Evans *et al.* 1965, Akhtar *et al.* 1975, Jerina *et al.* 1976). These reports were all with Gram negative bacteria. Therefore, questions were raised about the slow growing, acid-fast bacteria e.g. (i) what kinds of organisms were they? (ii) could they utilize anthracene for growth? (iii) what roles did they have in the mixed culture in degrading anthracene? In addition, as the slow growing, acid-fast bacteria were different from the Gram negative bacteria already reported in anthracene degradation, it was of interest to examine whether they use the same pathway as pseudomonds for anthracene degradation. This would be basically achieved, in part, by isolating and identifying metabolic intermediates and determining their specific stereochemistry. Therefore, pure culture studies of anthracene degradation by the slow growing bacteria were needed to answer these questions.

2. LITERATURE REVIEW.

2.1 Actinomycetes and their role in the environment.

Actinomycetes are a group of bacteria in the order Actinomycetales with the ability to form branching hyphae at some stage of their development. They are Gram positive bacteria with a high guanine (G) plus cytosine (C) content in their DNA (>55%) (Goodfellow and Cross 1984). They are commonly found in both natural and man-made environments. Most are strict saprophytes, but some form parasitic or mutualistic associations with plants and animals. This bacterial group comprises genera covering a wide range of morphology extending from the coccus (*Micrococcus*) and rod-coccus cycle (*Arthrobacter*), through fragmenting hyphal forms (*Nocardia*), to genera with a permanent and highly differentiated branched mycelium (*Micromonospora*, *Streptomyces*). Some but not all genera form spores which resist desiccation and mild heat (Goodfellow and Williams 1983).

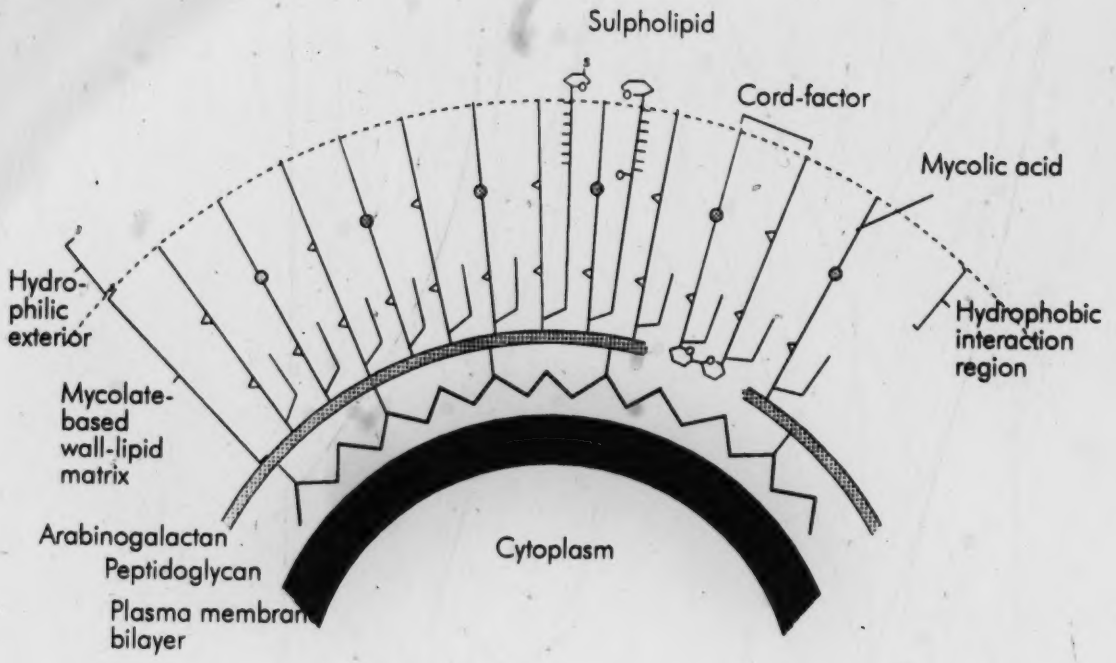
Actinomycetes are widely distributed in nature both in aquatic and terrestrial environments. Some plant, animal, and human disease-causing actinomycetes have been reviewed by Goodfellow and Williams (1983). For example, leaf gall in many plants is caused by *Rhodococcus fascians*, wilt and stunting of alfalfa by *Corynebacterium insidiosum*, equine pneumonia by *Rhodococcus equi*, diphtheria by *Corynebacterium diphtheriae*, tuberculosis by *Mycobacterium tuberculosis*, or leprosy by *Mycobacterium leprae*.

Soil is the most common source of actinomycetes. Many of them, mainly in the genus *Streptomyces*, produce useful secondary metabolites including antibiotics. Other genera in the *Corynebacterium-Mycobacterium-Nocardia* (CMN group) including *Rhodococcus* are known to be able to metabolize xenobiotic compounds. For example, there have been reports of tetralin and naphthalene degradation by *Corynebacterium* sp.

(Das Dua and Meera 1981, Sikkema and de Bont 1993); various kinds of PAH including compounds with greater than three benzene rings by *Mycobacterium* spp. (Guerin and Jones 1988, Heitkamp and Cerniglia 1989, Grosser *et al.* 1991, Kelly *et al.* 1991, Boldrin *et al.* 1993, Tiehm and Fritzsche 1995, Schneider *et al.* 1996). Other aromatic compounds such as methoxybenzoic acids, veratric acid, and phenols are degraded by *Nocardia* sp. (Trojanowski *et al.* 1977, Eggling and Sahm 1980), while biphenyl, polychlorinated biphenyls, ethylbenzene, nitroanisole, atrazine, dibenzothiophene, trichloroethylene, vinyl chloride, and other aromatic compounds are degraded by *Rhodococcus* spp. (Malachowsky *et al.* 1994, Warhurst and Fewson 1994, Seto *et al.* 1995, Nagy *et al.* 1995, Schafer *et al.* 1996, Seto *et al.* 1996, McKay *et al.* 1997).

The CMN group of bacteria have cell envelopes that contain α-branched β-hydroxylated long-chain fatty acids, known as mycolic acids as the major constituent of the cell wall (Minnikin and Goodfellow 1980). These mycolic acids were originally characterized from *Mycobacterium tuberculosis* and later found in a number of taxa that have an arabinogalactan in their walls and a peptidoglycan based on *meso*-diaminopimelic acid. The mycolic acids are varied in chain length among the CMN group bacteria and are covalently bound to an arabinogalactan polymer, which in turn, is covalently bound to peptidoglycan (McNeil *et al.* 1990). Mycolic acid carbon chain length ranges from 22 to 36 in the genus *Corynebacterium* to 60 to 90 in the genus *Mycobacterium* (Goodfellow 1992). Figure 1 shows a diagram of the generalized mycobacterial cell wall which contains mycolic acids as its components (Minnikin and O'Donnell 1984). The mycolic acids found in the CMN bacteria group confer the hydrophobic properties of the cell surface. This hydrophobicity is important in microbial degradation of various toxic compounds that are themselves hydrophobic as it enables the bacteria to adhere to hydrophobic carbon substrates which may allow the direct utilization of those substrates.

Fig. 1 A model of the mycobacterial cell wall illustrating mycolic acids covalently bound to peptidoglycan-linked arabinogalactan. Unsaturations, whether *cis* or *trans* double bonds or cyclopropane rings, are represented by triangles and oxygen functions are indicated by circles (after Minnikin and O'Donnell 1984).

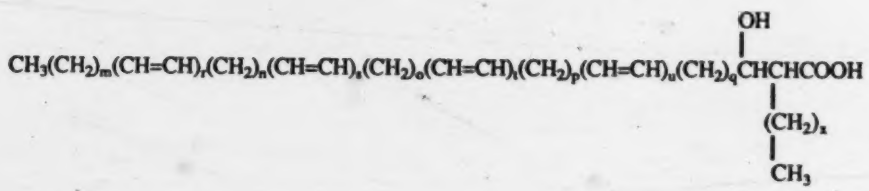

Sulpholipid
Cord-factor
Mycolic acid
Hydro-
philic
exterior
Hydrophobic
interaction
region
Mycolate-
based
wall-lipid
matrix
Arabinogalactan
Peptidoglycan
Plasma membran
bilayer
Cytoplasm

2.2 Rhodococci and some of their roles in the environment.

2.2.1 Characteristics of rhodococci.

Rhodococci are aerobic, nonsporulating, Gram positive, nonmotile, chemoorganotrophic bacteria that belong to the soil actinomycetes. They normally exhibit a growth cycle ranging from cocci or short rods to more complex growth phases that form filaments with short projections, elementary branching or extensively branched hyphae. These bacteria are partially acid-fast with an oxidative type of metabolism (Goodfellow 1989). Strains belonging to the genus *Rhodococcus* have a cell wall peptidoglycan chemotype IV which contains major amounts of *meso*-diaminopimelic acid, arabinose and galactose (Goodfellow 1992). Differentiation of rhodococci from the other members of mycolic acid-containing actinomycetes at the genus level relies mainly on chemotaxonomy (Goodfellow 1992). Their cell envelopes contain straight-chain, saturated and unsaturated fatty acids and tuberculostearic acid (10-methyloctadecanoic acid) associated with mycolic acids (Minnikin and O'Donnell 1984).

Shown below is the general structural formula of mycolic acids which are generally found in nocardia, corynebacteria, and rhodococci but not mycobacteria (Goodfellow *et al.* 1973) which then are called the non-mycobacterial mycolic acids.



The value of r, s, t and u are between 0 and 4, and m, n, o, p and q are all greater than 1. No values were given for x but it is assumed to be a long chain fatty acid.

Examination of whole-cell methanolysates for the presence of mycolic acids is the first step in chemotaxonomy. This is a simple but reliable test in differentiating these mycolic acid containing bacteria. The chemotaxonomic characters of mycolic acids can be examined easily using a thin-layer chromatographic technique devised by Minnikin *et al.* (1975). The non-mycobacterial mycolic acids lack oxygen functions whereas the mycolic acids from mycobacteria contain components with oxygen functions, e.g. keto, methoxy, and carboxylic acid, in addition to the β-hydroxyacid unit. Therefore, thin-layer chromatography of the methyl esters of the total mycolic acids from nocardia, corynebacteria, and rhodococci give a single spot pattern of mycolates and those of mycobacteria give a multispot pattern (Minnikin 1982; Minnikin and Goodfellow 1980; Minnikin *et al.* 1975).

After mycolic acids have been found in the bacteria, their esters can be isolated and examined by pyrolysis gas chromatography, mass spectrometry of the intact esters, gas chromatography of trimethylsilyl ether derivatives, and high-performance liquid chromatography of bromophenacyl derivatives. Members of the genus *Rhodococcus* generally have mycolic acids that contain 34-64 carbon atoms and upon pyrolysis gas chromatography, their methyl esters yield fatty acid esters with carbon chains between 12-18 carbon atoms (Goodfellow 1992). Criteria which help differentiate mycolic acid-containing genera are summarized in Table 1.

The taxonomic positions of rhodococci have undergone frequent revisions as false names are corrected, existing strains are reclassified into newly recognized genera, *Gordona* and *Tsukamurella*, and new species are identified (Finnerty 1992).

Table 1 Differential characteristics of the genus *Rhodococcus* and other wall chemotype IV actinomycetes containing mycolic acids (after Goodfellow 1989, 1992).

Characteristics	*Gordona*	*Coryne-bacterium*	*Myco-bacterium*	*Nocardia*	*Rhodo-coccus*	*Tsuka-murella*
Morphological characters						
Substrate mycelium	-	-	D	+	D	-
Aerial mycelium	-	-	D	D	-	-
Conidia	-	-	-	D	-	-
Lipid characters						
Fatty acids						
Tuberculostearic acid	+	-	+	+	+	+
Phospholipids						
Phosphotidylethanolamine	+	-	+	+	+	+

Table 1 (Continued).

Characteristics	*Gordona*	*Coryne-bacterium*	*Myco-bacterium*	*Nocardia*	*Rhodo-coccus*	*Tsuka-murella*
Predominant menaquinone	MK-9(H_2)	MK-8(H_2) or MK-9(H_2)	MK-9(H_2)	MK-8(H_2) or MK-9(H_2)	MK-8(H_2) or MK-9(H_2)	MK-9
Mycolic acids						
Overall size (number of carbons)	48-66	22-38	60-90	44-60	34-64	64-78
Number of double bonds	1-4	0-2	1-3	0-3	0-2	1-6
Ester released on pyrolysis (number of carbons)		8-18	22-26	12-18	12-16	20-22
GC content (mol %)	63-69	51-59	62-70	64-72	63-73	67-78

Symbols: +, 90% or more of the strains are positive

-, 90% or more of the strains are negative

D, different reactions occur in different taxa (species of a genus or genera of a family)

2.2.2 Rhodococci and their involvement in xenobiotic transformations.

Bacteria in the genus *Rhodococcus* are are widely distributed in nature but only rarely encountered as primary pathogens in healthy individuals (Goodfellow 1992). They have been isolated from a wide range of environments including soil, fresh water, marine habitat, and activated sludge (Goodfellow and Williams 1983; Warhurst and Fewson 1994). These organisms have the ability to transform a wide range of xenobiotic compounds such as halogenated phenols and alkanes, substituted benzenes, anilines, and quinolines. Thus they may play an active role in the biodegradation of toxic pollutants in soil.

Rhodococci are autochthonous, that is always present in an environment, despite their slow growth. Pseudomonads and other Gram negative bacteria generally exhibit catabolite repression, in the presence of an easily-degradable substrate leading to suppression of the breakdown of other substrates. Rhodococci generally do not appear to exhibit this behavior. In several cases the presence of glucose actually accelerates the metabolism of other compounds such as aromatics (Appel *et al.* 1984, Fuchs *et al.* 1991).

Finnerty (1992) has reviewed the degradation of xenobiotic compounds by rhodococci which show a broad substrate diversity for the degradation of various compounds such as phenols, aromatic acids, halogenated phenols, halogenated alkanes, substituted benzenes, anilines, and quinolines. For enviromental applications, rhodococci have been employed in bioremediation of chlorinated hydrocarbons and phenolics. For example, *Rhodococcus chlorophenolicus*, a chlorophenol-degrading actinomycete, has been used for the bioremediation of chlorophenols in contaminated soil (Apajalahti and Salkinoja-Salonen 1986, Apajalahti *et al.* 1986) and chlorophenol-containing ground water (Valo *et al.* 1990). This bacterium efficiently degraded chlorinated phenols ranging from dichloro- to pentachlorophenols. The reaction involves total dechlorination prior to ring

cleavage rather than ring cleavage and then dechlorination, as described for other microorganisms degrading mono- and dichlorinated phenols (Finnerty 1992).

There has been a report of a *Rhodococcus* sp. which plays a role as a member of the bacterial consortium degrading alicyclic hydrocarbons (Lloyd-Jones and Trudgill 1989), where *Rhodococcus*, *Pseudomonas*, and *Flavobacterium* were isolated from an oil refinery site. Individually, the bacteria could not utilize alicyclic hydrocarbons as a growth substrate. However, when these bacteria were present as a three-member consortium, they could grow on a wide range of alicyclic hydrocarbons.

Rhodococcus spp. have been reported capable of degrading a wide range of polychlorinated biphenyls (Masai *et al.* 1995, McKay *et al.* 1997). Unlike most Gram negative polychlorinated biphenyl degraders which degrade a limited range of polychlorinated biphenyls, *Rhodococcus* spp. (such as the strain RHA1) can efficiently transform 45 components of the 62 major peaks of polychlorinated biphenyl mixture of Kanechlors (Seto *et al.* 1995, 1996). Members of the genus *Rhodococcus* can catalyze a highly specific carbon-sulfur bond cleavage reaction of organic sulfur-containing heterocycles such as dibenzothiophene and can use dibenzothiophene as a sole sulfur source (Kilbane and Jackowski 1992, Gallagher *et al.* 1993).

In the case of PAH degradation, Warhurst and Fewson (1994) reviewed the biotransformation of hydrocarbons by the genus *Rhodococcus*. *Rhodococcus* has been shown capable of utilizing various PAHs as growth substrate e.g. naphthalene (Grunde *et al.* 1992, Malachowsky *et al.* 1994); biphenyl (Malachowsky *et al.* 1994); pyrene, phenanthrene, anthracene, fluoranthene, and chrysene (Walter *et al.* 1991). Recently Bouchez and colleagues (1996) employed three strains of rhodococci in their study on microbiological fate of PAHs. Each strain reportedly was able to utilize either naphthalene, fluoranthene, or pyrene as the sole carbon and energy source.

2.3 Polycyclic aromatic hydrocarbons (PAHs).

2.3.1 Structure, sources, and distribution of PAHs.

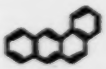
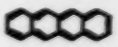
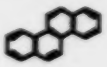
PAHs are neutral, nonpolar organic molecules that comprise two or more benzene rings arranged in various configurations. These compounds originate from a variety of sources such as petroleum, coal tar, wood-preserving creosote, incomplete combustion of fossil fuels, and forest fires. PAHs are the major components in coal tar creosote: they represent approximately 85-90% of creosote constituents (Mueller *et al.* 1989). A simplified list of major PAHs found in creosote based on field data obtained from both creosote-contaminated soil and water is shown in Table 2. Industrial effluents from coal gasification and liquification processes, waste incineration, coke, carbon black, and other petroleum-derived products also contribute to PAHs in terrestrial, aquatic and marine sediments. PAHs enter the biosphere through various routes such as accidental discharges of fossil fuels, direct aerial fallout, chronic leakage, industrial and sewage disposal, and surface water runoff. Due to their poor aqueous solubility, PAHs released into the environment are found deposited in, and bound to, soil and eventually enter the ground water. Some are washed off by rain to lakes and rivers where they also become bound to particles and subsequently deposited in the sediments of river, lake, and marine waters (Edwards 1983, Dzombak and Luthy 1984). There are concerns about the presence of PAHs in air, soil, and water systems because of their toxicity and potential mutagenic and carcinogenic effects (IARC 1983, Cerniglia and Heitkamp 1989).

A summary of chemical structures, solubility, carcinogenicity, and pollutant designation of PAHs of environmental interest is given in Table 3.

Table 2 Major PAHs present in coal tar creosote (after Mueller *et al.*, 1989).

Compound	Molecular Weight	Aqueous solubility (mg/L, 25°C)
Naphthalene	128.2	31.7
2-Methylnaphthalene	142.2	25.4
Phenanthrene	178.2	1.3
Anthracene	178.2	0.07
1-Methylnaphthalene	142.2	28.5
Biphenyl	154.2	7.5
Fluorene	166.2	2.0
2,3-Dimethylnaphthalene	156.2	3.0
2,6-Dimethylnaphthalene	156.2	2.0
Acenaphthene	154.2	3.9
Fluoranthene	202.3	0.26
Chrysene	228.2	0.002
Pyrene	202.3	0.14
Anthraquinone	208.2	-
2-Methylanthracene	192.3	0.04
2,3-Benzo[b]fluorene	216.3	0.002
Benzo[a]pyrene	252.3	0.003

Table 3 Chemical structures, formulae and pollutant designation of some PAHs (after Dzombak and Luthy 1984).

Compound	molecular structure	molecular formula	pollutant list*
Naphthalene		$C_{10}H_8$	E
Biphenyl		$C_{12}H_{10}$	
Acenaphthene		$C_{12}H_{10}$	E
Fluorene		$C_{13}H_{10}$	E
Anthracene		$C_{14}H_{10}$	E
Phenanthrene		$C_{14}H_{10}$	E
Fluoranthrene		$C_{16}H_{10}$	E, W
Pyrene		$C_{16}H_{10}$	E
Benz(a)anthracene		$C_{18}H_{12}$	E
Naphthacene		$C_{18}H_{12}$	
Chrysene		$C_{18}H_{12}$	E

* PAHs on Environmental Protection Agency (E) and World Health Organization (W) pollutant list.

A variety of processes including volatilization, adsorption, chemical oxidation, photo-decomposition, and biodegradation all contribute to the environmental loss of PAHs. There have been many attempts to use microorganisms to degrade PAHs because microbial mineralization does not create other new problems as may occur with physical or chemical treatments. Eucaryotic microorganisms such as fungi cannot use PAHs for growth but usually cometabolize the PAHs to dead-end products, some of which are probably more hazardous than the parent compounds. In contrast, bacteria, as procaryotic organisms can completely degrade many PAHs and use them as the sole carbon and energy source for growth (Cerniglia and Heitkamp 1989). The main problem for bacteria to overcome for PAH removal is their low solubility and therefore low bioavailability.

2.3.2 Effects of bioavailability and solubility on PAH biodegradation.

So far there has been no evidence that crystalline PAHs are available for microbial degradation. These poorly soluble compounds are only available for microorganisms when they are in the dissolved state. The low water solubility of PAHs and the low dissolution rate of their crystals mean that microbial growth is often substrate-limited even in the presence of excess crystalline PAHs causing the degradation rate to depend on the dissolution rate (Tiehm and Fritzsche 1995). The dissolution rate is influenced by physical and chemical factors such as the solubility of the specific compound, mixing rate, and particle surface area, and it can be increased by some means such as using organic solvents, surfactants, decreasing the particle size thus increasing the surface area, or increasing the stirring rate (Boldrin *et al.* 1993).

Since the availability of PAHs to microorganisms is also limited by their low water solubility, there have been numerous reports of attempts to use surfactants to enhance the bioavailability of PAHs. From the literature reviewed so far it has been shown that the

effects of surfactants on biodegradation are variable, ranging from inhibition of degradation to no effect to stimulation of degredation (Laha and Luthy 1991, Tiehm 1994, Liu *et al.* 1995, Providenti *et al.* 1995, Volkering *et al.* 1995, Soeder *et al.* 1996). The variable results of the effect of surfactants has been attributed to several factors such as the types of surfactants, concentrations, types of PAH, strains of microorganisms, and the environmental conditions of microbial growth. The interactions among these factors are complex and should be evaluated in order to assess whether surfactants may be used beneficially for enhancement of bioremediation of contaminated soils and sediments (Laha and Luthy 1992).

2.4 Microbial degradation of PAHs.

Microorganisms are important in the degradation of aromatic hydrocarbons in terrestrial and aquatic ecosystems. Biodegradation of the compounds can be considered on the one hand as part of the normal process of the carbon cycle, and on the other hand as the removal of pollutants from the environment (Smith 1990). Evidence of microbial degradation of PAHs can be dated back to about 70 years ago. There was a report in 1927 that W.O. Tausson found three kinds of bacteria in soils from the Black Sea oil fields that could metabolize naphthalene and in the next year Grey and Thornton isolated several strains of bacteria from naphthalene-treated soils that could use naphthalene as a carbon source (Walker and Wiltshire 1953). Since then there have been numerous studies relating to degradation of PAHs by microorganisms as shown in Table 4.

Both prokaryotic and eukaryotic microorganisms have the enzymatic capacity to oxidize a wide range of PAHs. An aromatic compound is considered to be biodegraded if the ring undergoes cleavage. Bacteria oxidize PAHs to dihydroxylated compounds as a prelude to ring fission and assimilation whereas fungi hydroxylate PAHs as a prelude to detoxification (Dagley 1981). There is no evidence yet that fungi can use PAHs as the

Table 4 Some bacteria and fungi which are involved in PAH degradation.

Organisms	PAHs	References
Gram negative bacteria:		
Aeromonas sp.	Naphthalene and phenanthrene	Kiyohara and Nagano 1978
Alcaligenes denitrificans	Fluoranthene	Weissenfels *et al.* 1990
Beijerinckia sp.	Benzo[a]pyrene and benz[a]-anthracene	Gibson *et al.* 1975
	Phenanthrene	Strandberg *et al.* 1986
Flavobacterium sp.	Phenanthrene	Foght *et al.* 1990
Pseudomonas aeruginosa	Anthracene and phenanthrene	Evans *et al.* 1965
Pseudomonas cepacia	Naphthalene, phenanthrene, anthracene, fluorene, dibenzothiophene, biphenyl, dibenzofuran, acenaphthene, acenaphthylene, 2,3-dimethylnaphthalene, and 2,6-dimethylnaphthalene	Grifoll *et al.* 1995
Pseudomonas fluorescens	Chrysene, benz[a]anthracene, and benzo[b]naphthothiophene	Caldini *et al.* 1995
	Anthracene and phenanthrene	Menn *et al.* 1993
Pseudomonas paucimobilis	Phenanthrene	Weissenfels *et al.* 1990
Pseudomonas putida	Naphthalene	Jeffrey *et al.* 1975
	Anthracene and phenanthrene	Gibson *et al.* 1975
	Fluoranthene and	Barnsley 1975a

Table 4 (Continued)

Organisms	PAHs	References
	benzo[a]pyrene	
	Phenanthrene and anthracene	Sanseverino *et al.* 1993
Pseudomonas stutzeri and *Pseudomonas saccharophila*	Phenanthrene	Stringfellow and Aitken 1994
Pseudomonas vesicularis	Fluorene	Weissenfels *et al.*1990
Sphingomonas yanoikuyae	1,2-Dihydronaphthalene	Eaton *et al.* 1996
Xanthomonas sp.	Pyrene, benzo[a]pyrene, and carbazole	Grosser *et al.* 1991
Gram positive bacteria:		
Arthrobacter sp.	Fluorene	Casellas *et al.* 1997 Grifoll *et al.* 1992
Corynebacterium renale	Naphthalene	Das Dua and Meera 1981
Mycobacterium sp.	Pyrene	Jimenez and Bartha 1996
	Pyrene, benzo[a]pyrene	Grosser *et al.* 1991
	Benz[a]anthracene, and benzo[a]pyrene	Schneider *et al.* 1996
	Phenanthrene, fluorene, fluoranthene, and pyrene	Boldrin *et al.* 1993, and Tiehm and Fritzsch 1995
Nocardia sp.	Naphthalene	Trecanni *et al.* 1954

Table 4 (Continued)

Organisms	PAHs	References
Rhodococcus spp.	Naphthalene	Grund *et al.* 1992
	Naphthalene, anthracene, phenanthrene, fluorene, fluoranthene, pyrene, and chrysene	Walter *et al.* 1991
	Napthalene, fluoranthene, and pyrene	Bouchez et.al. 1996
Staphylococcus auriculans	Fluorene	Monna *et al.* 1993
Streptomyces flavovirens	Phenanthrene	Sutherland *et al.* 1990
Cyanobacteria:		
Agmenellum quadruplicatum	Phenanthrene	Narro *et al.* 1992a
Oscillatoria sp.	Naphthalene	Narro *et al.* 1992b
Molds:		
Coriolopsis polyzona	Anthracene	Vyas *et al.* 1994
Crinipellis stipitaria	Pyrene	Lambert *et al.* 1994
		Lange *et al.* 1994
Cunninghamella elegans	Phenanthrene	Cerniglia *et al.* 1989
		Sutherland *et al.* 1993
	Benz[a]anthracene	Cerniglia *et al.* 1994
	Fluorene	Pothuluri *et al.* 1993

Table 4 (Continued)

Organisms	PAHs	References
	Anthracene and phenanthrene	Cerniglia and Yang 1984
Penicillium spp.	Chrysene	Kiehlmann *et al.* 1996
Phanerochaete chrysosporium	Phenanthrene	Sutherland *et al.* 1991
	Anthracene	Vyas *et al.* 1994
Pleurotus ostreatus	Anthracene	Vyas *et al.* 1994
	Phenanthrene	Bezalel *et al.* 1996a
	Pyrene, anthracene, fluorene, and dibenzothiophene	Bezalel *et al.* 1996b
Trametes vesicolor	Anthracene	Vyas *et al.* 1994
	Anthracene and benzo[a]pyrene	Collins *et al.* 1996
Syncephalastrum racemosum	Phenanthrene	Sutherland *et al.* 1993
	Chrysene	Kiehlmann *et al.* 1996
Yeasts:		
Saccharomyces cerevisiae	Anthracene and pyrene	Torres *et al.* 1995
Trichosporon penicillatum	Phenanthrene	MacGillivray and Shiaris 1993

sole source of carbon and energy; but when grown on an alternative carbon source they often produce enzymes to oxidize PAHs.

In his review of the literature, Cerniglia (1992) summarized general statements about microbial degradation of PAHs, some of which are given as follows. Microbes, both procaryotes and eucaryotes, have the ability to metabolize PAHs and play an important role in PAH degradation in terrestrial and aquatic environments. Generally, the rate of PAH degradation is inversely proportional to the number of rings in the PAH molecule thus the lower molecular weight PAHs are biodegraded more rapidly than the higher molecular weight compounds. Microorganisms, both prokaryotes and eukaryotes, require the presence of molecular oxygen to initiate the enzymatic attack on the PAH rings in their biodegradation mechanisms. Different routes of initial oxidation of PAHs by microorganisms are shown in Fig. 2.

Bacteria use dioxygenase enzymes to catalyze the incorporation of both atoms of the oxygen molecule into the aromatic nucleus. This reaction is the major mechanism for the bacterial, initially oxidative, attack on PAHs that leads to the formation of dihydrodiols which are in the *cis*-configuration. *Cis*-dihydrodiols are stereoselectively dehydrogenated by *cis*-dihydrodiol dehydrogenases, which rearomatizes the nucleus to form dihydroxylated intermediates. Subsequent enzymatic fission of the aromatic ring is also catalyzed by highly regio- and stereoselective dioxygenases by which the ring is cleaved using either the *ortho* or *meta* pathway, depending on the type of microorganism.

A diverse group of lignolytic and non-lignolytic fungi also have the ability to oxidize PAHs. Non-lignolytic fungi utilize cytochrome P450 monooxygenases to catalyze the incorporation of one atom of molecular oxygen into the aromatic nucleus and reduce the remaining atom to water. The resulting arene oxide intermediate can undergo further metabolism by epoxide hydrolase to form a dihydrodiol with the *trans*-configuration or can undergo nonenzymatic rearrangement to form a phenol, which can be conjugated with

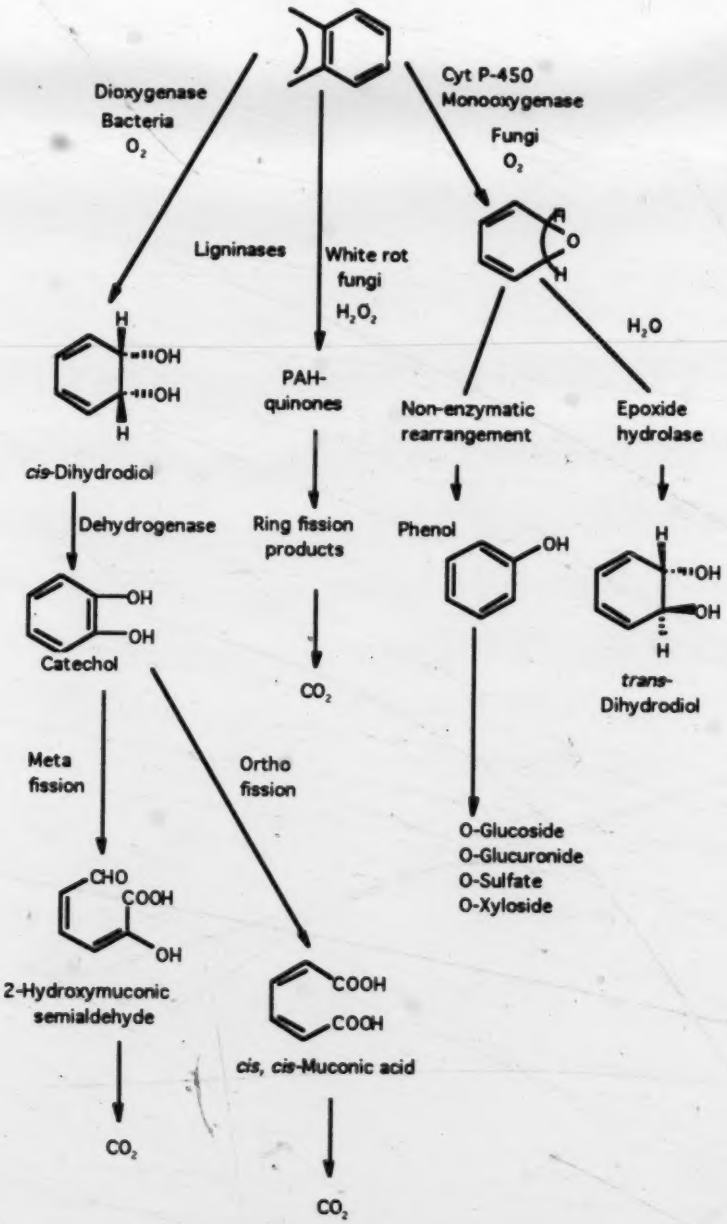


Fig. 2 Pathways for the microbial metabolism of polycyclic aromatic hydrocarbons (after Pothuluri *et al.* 1995).

sulfate, glucuronic acid, or glucose. These reactions are similar to those occuring in fish and mammals. Lignolytic fungi produce extracellular lignin-degrading enzymes, ligninases, that not only degrade lignin-related compounds but also catalyze the one-electron oxidation of PAHs to quinones. There has been evidence of ring cleavage products and mineralization by *Phanerochaete chrysosporium* from anthracene degradation (Hammel *et al.* 1991) and from phenanthrene degradation (Hammel *et al.* 1992, Moen and Hammel 1994).

2.4.1 Pathways of anthracene metabolism.

From research on various bacteria over the years, the complete degradative pathways for the oxidation of anthracene and phenanthrene have been elucidated for some bacteria.

For anthracene, a number of investigators have been working on bacterial strains of *Flavobacterium*, *Pseudomonas*, and *Beijerinckia* (Akhtar *et al.* 1975, Dagley and Gibson 1965, Evans *et al.* 1965, Jerina *et al.* 1976, Menn *et al.* 1993) and the metabolic pathway of anthracene degradation has been proposed as shown in Fig. 3. Anthracene is initially oxidized in the 1,2-position to form (+)-*cis*-1R,2S-dihydroxy-1,2-dihydroanthracene (anthracene *cis*-1,2-dihydrodiol). The next step is the conversion of anthracene *cis*-1,2-dihydrodiol to 1,2-dihydroxyanthracene which is enzymatically cleaved by a dioxygenase to yield *cis*-4-(2-hydroxynaphth-3-yl)-2-oxo-but-3-enoic acid. Further metabolism of this ring fission product leads to 2-hydroxy-3-naphthoic acid which is subsequently metabolized through salicylate and catechol by the enzymes of the naphthalene pathway.

Fungi oxidize anthracene primarily in the 1,2-position to form *trans*-1S,2S-dihydroxy-1,2-dihydroanthracene (anthracene *trans*-1,2-dihydrodiol) as shown in Fig. 4. This dihydrodiol can be further conjugated with sulfate or glycosides (Cerniglia 1982, Cerniglia and Yang 1984, Sutherland *et al.*1992).

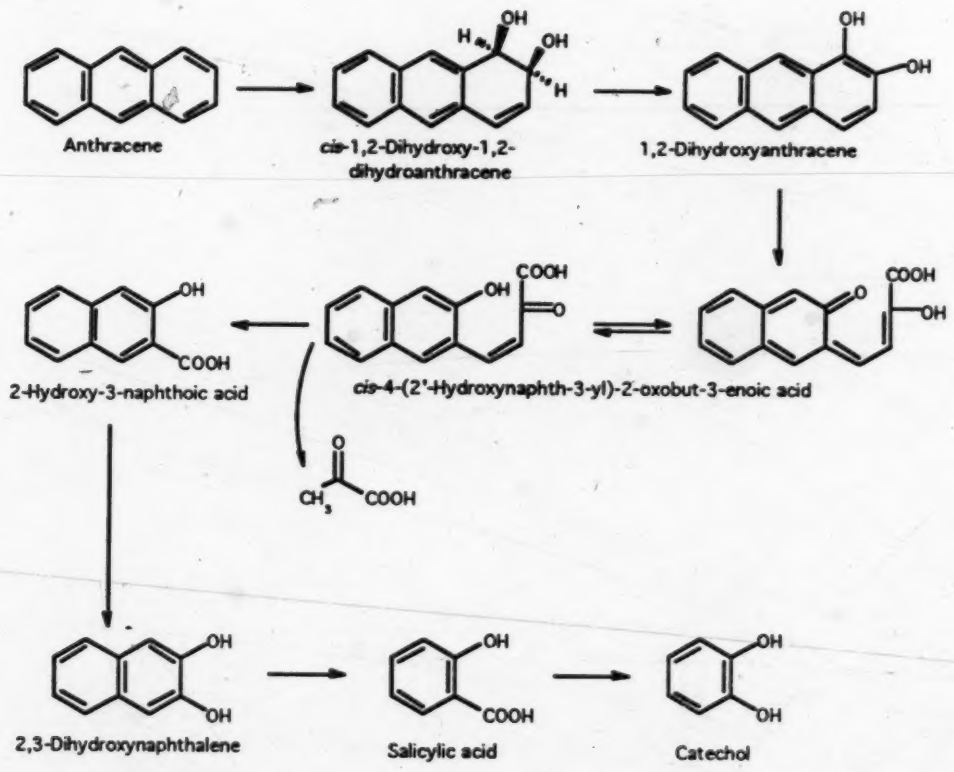


Fig. 3 The pathway for the bacterial oxidation of anthracene (after Varanasi 1989).

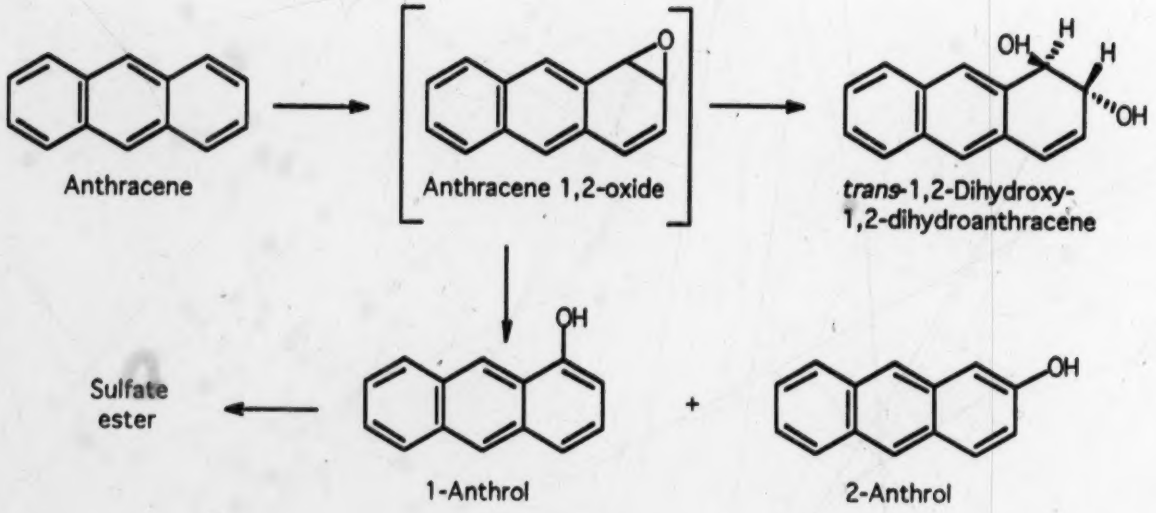


Fig. 4 Initial reactions in the oxidation of anthracene by fungi (after Atlas 1984).

2.4.2 Pathways of phenanthrene metabolism.

Bacteria reportedly metabolize phenanthrene by two different pathways as shown in Fig. 5 (Evans *et al.* 1965, Jerina *et al.* 1976, Kiyohara and Nagao 1978, Barnsley 1983, Menn *et al.* 1993). The initial sites of enzymatic attack are in the 1,2- and 3,4- positions to form (+)-*cis*-1R,2S-dihydroxy-1,2-dihydrophenanthrene (phenanthrene *cis*-1,2-dihyrodiol) and (+)-*cis*-3S,4R-dihydroxy-3,4-dihydrophenanthrene (phenanthrene *cis*-3,4-dihydrodiol) of which the latter is the major isomer formed (>90%). *Pseudomonas* and *Nocardia* strains oxidized phenanthrene *cis*-3,4-dihydrodiol to 3,4-dihyroxphenanthrene which is subsequently cleaved and converted to 1-hydroxy-2-naphthoic aicd. This ring cleavage product is oxidatively decarboxylated to 1,2-dihydroxynaphthalene which can then enter the naphthalene pathway. *Aeromonas*, *Vibrio*, *Alcaligenes*, and *Micrococcus* strains use an alternate pathway for 1-hydroxy-2-naphthoic acid catabolism. They oxidize 1-hydroxy-2-naphthoic acid via *ortho*-phthalic acid to protocatechuic acid.

Fungi metabolize phenanthrene using cytochrome P450 monooxygenases to phenanthrene *trans*-1,2-, *trans*-3,4-, and *trans*-9,10-dihydrodiols, and a glycoside conjugate (Cerniglia and Yang 1984, Cerniglia *et al.* 1989, Cerniglia 1992, Sutherland *et al.* 1993) (Fig. 6). Thus there are different routes for the initial oxidation of phenanthrene. Non-lignolytic fungi and lignolytic fungi grown under nonlignolytic conditions utilize cytochrome P450 monooxygenases to form these dihydrodiols in the *trans*-configurations. Interestingly, there have been reports recently that lignolytic fungi used a different pathway to metabolize phenanthrene. In phenanthrene degradation by *Phanerochaete chrysosporium*, the fungus oxidized phenanthrene at the 9,10- position to give 2,2'-diphenic acid as a ring cleavage product (Hammel *et al.* 1992, Moen and Hammel 1994).

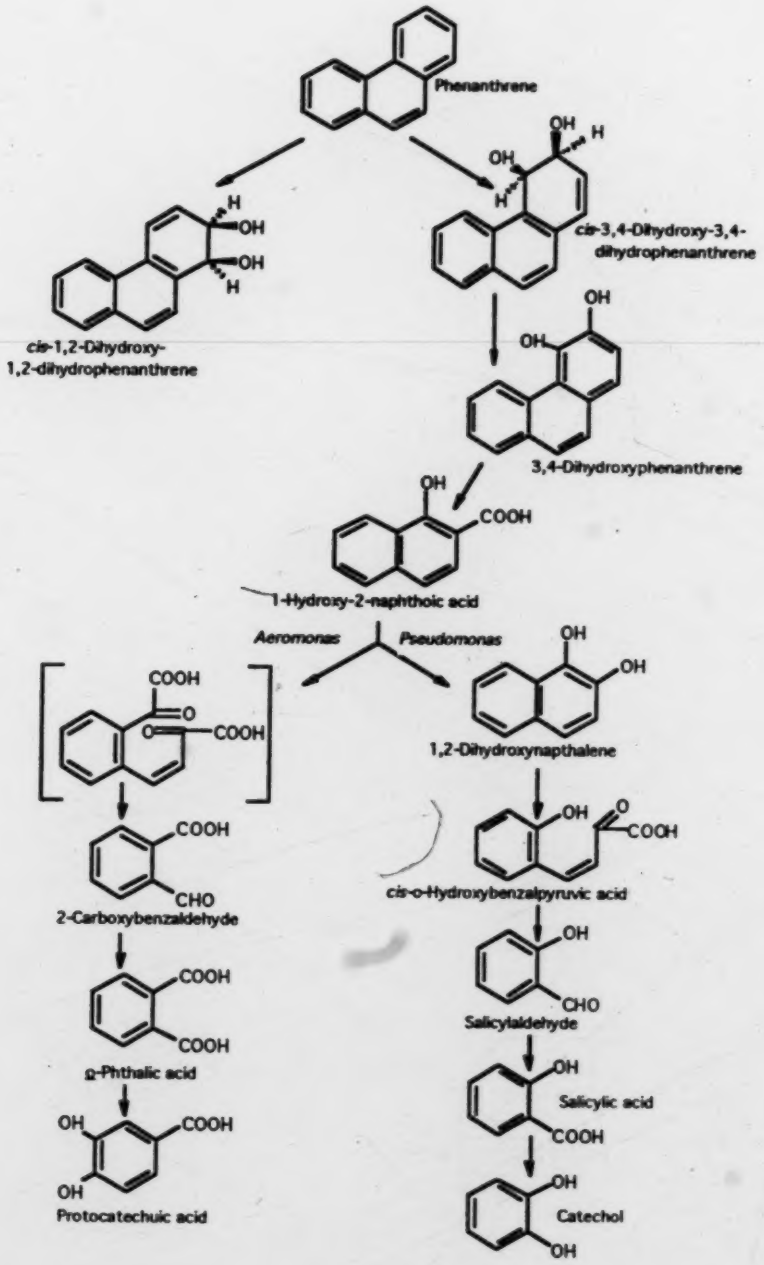


Fig. 5 The pathway for the bacterial oxidation of phenanthrene (after Varanasi 1989).

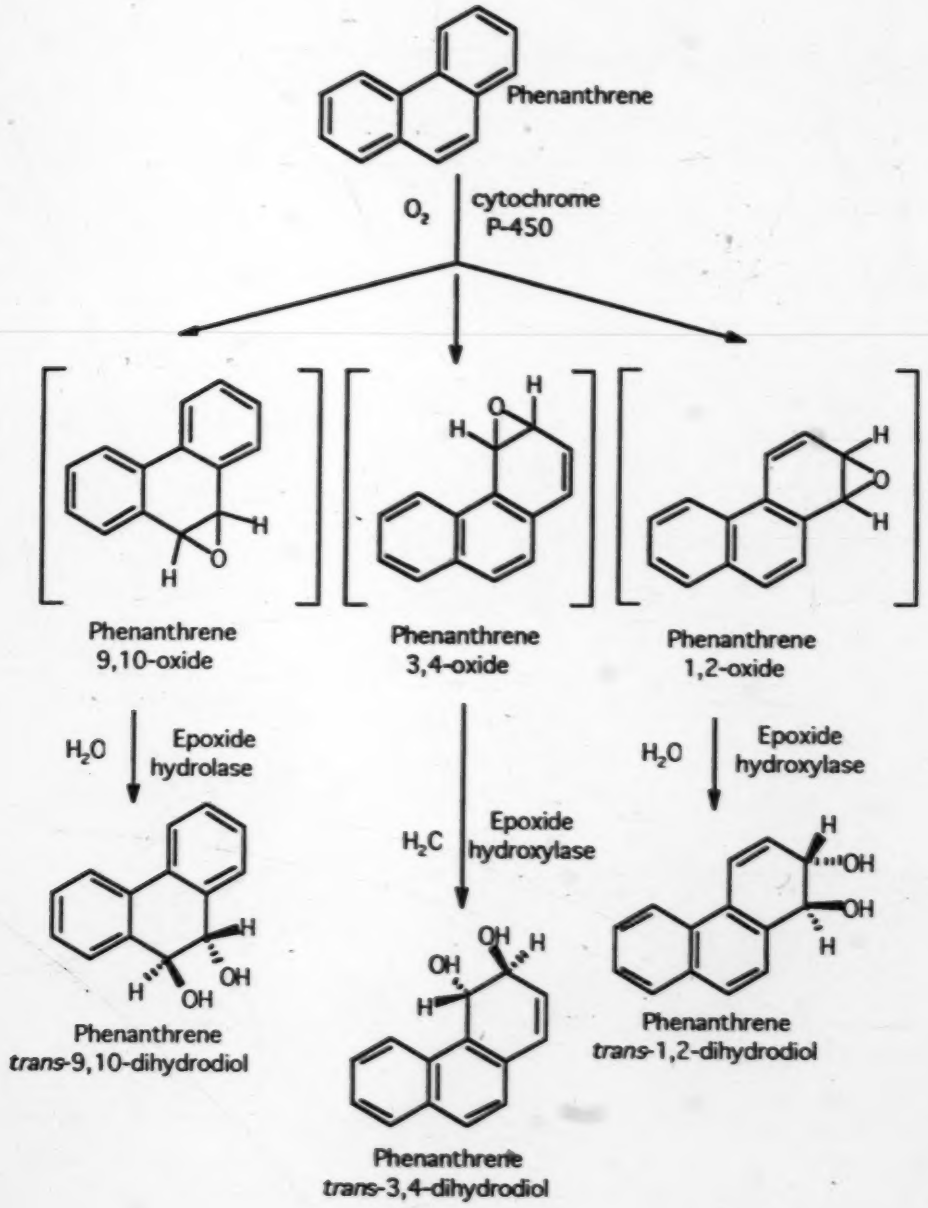


Fig. 6 Fungal metabolism of phenanthrene via cytochrome P450 monooxygenase (after Sutherland *et al.* 1993).

2.4.3 Ligninase involvement in PAH degradation.

White-rot fungi are wood-decaying basidiomycetes which normally produce lignin peroxidase, a haem-containing enzyme with protoporphyrin IX that catalyzes the one-electron oxidation of various lignin-related substrates (Kirk and Farrell 1987). These fungi are the only known organisms which are capable of degrading lignin extensively to carbon dioxide and water in pure culture (Gold and Alic 1993). Since lignin contains a variety of bonds that are commonly present in aromatic pollutants and the lignin-degrading system of these fungi is nonspecific and oxidative, attention has been focused on these fungi for their ability to degrade toxic aromatic pollutants (Gold and Alic 1993). It has been shown that the white-rot fungi that break down the lignin of wood can also mineralize a wide variety of aromatic pollutants, including certain PAHs, under culture conditions that promote the expression of lignolytic enzymes (Bumpus *et al.* 1985, Bumpus 1989). The ability of these fungi to cleave carbon-carbon bonds in PAHs is unique among eucaryotes because ring fission of PAHs has been considered previously as an exclusively bacterial process (Gibson and Subramanian 1984).

Hammel *et al.* (1991) reported that under lignolytic conditions *P. chrysosporium* oxidized anthracene mainly to phthalate and carbon dioxide via anthraquinone and that these reactions were probably mediated by lignin peroxidase and other enzymes of lignolytic metabolism. This oxidation process differs from those in bacteria which proceeds via anthracene *cis*-1,2-dihydrodiol to yield salicylate rather than phthalate as a ring cleavage product (Cerniglia and Heitkamp 1989). Likewise, phenanthrene was shown to be oxidized to a ring-fission product, 2,2'-diphenic acid, via phenanthrene-9,10-quinone and mineralization also occured (Hammel *et al.* 1992). This phenanthrene oxidation was further investigated by Moen and Hammel (1994) who then reported that another ligninolytic enzyme, manganese peroxidase, was involved in the oxidation of phenanthrene by this fungal strain. Manganese peroxidase is a manganese-dependent lignin peroxidase

which oxidizes Mn(II) to Mn(III), which in turn oxidizes the lignin-related substrates (Kirk and Farrell 1987). Lignin peroxidases have been reportedly involved in the degradation of other PAHs including benzo[a]pyrene (Sanglard *et al.* 1986) and pyrene (Hammel *et al.* 1986).

Thus, it has been shown that white-rot fungi are able to oxidize some PAHs using either lignin peroxidase or manganese peroxidase leading to ring cleavage products. In addition to peroxidases, many white-rot fungi except *P. chrysosporium* produce an extracellular laccase. Laccase is a blue copper containing phenol oxidase which catalyzes the one-electron oxidation of phenols to phenoxy radicals (Gold and Alic 1993). Laccase can catalyze the alkyl-phenyl and C_α-C_β cleavage of phenolic lignin dimers (Kirk *et al.* 1987, Higuchi 1990). Recently, Collins *et al.* (1996) reported that laccases from *Trametes vesicolor* were able to catalyze the *in vitro* oxidation of anthracene and benzo[a]pyrene. This indicated that laccases may have a role in the oxidation of PAHs by white rot fungi.

2.5 Enzymatic oxidation of PAHs.

2.5.1 Bacterial oxygenases.

As previously stated, oxygenase enzymes play important roles in the microbial degradation of PAHs by catalyzing the insertion of molecular oxygen into PAH molecules. Mason and Cammack (1992) have reviewed the litertature on oxygenases, especially bacterial dioxygenases. Oxygenases can be classified into two groups: monooxygenases and dioxygenases.

Monooxygenases incorporate one atom of molecular oxygen into one molecule of substrate and the other atom is reduced to water. Dioxygenases catalyze the incorporation of both atoms of oxygen into a molecule of substrate. These enzymes are involved at

several stages in the catabolic pathways of PAHs and can be divided into two groups. The first group includes dioxygenase enzymes involving in ring hydroxylation. These enzymes require reduced cofactors, either NADH or NADPH, in addition to oxygen. They dihydroxylate aromatic substrates to *cis*-diols. The second group includes dioxygenase enzymes involving in ring fission. These enzymes have no cofactor requirement and cleave the benzene ring of hydroxylated aromatic substrates.

Ring hydroxylating enzymes have been extensively investigated because the enzymes play such an important role in the initial oxidation of aromatic compounds. The enzymes are nonheme dioxygenases that oxidize aromatic substrates to give *cis*-dihydrodiols. All are soluble, multicomponent enzymatic systems comprising three protein components: a flavoprotein, a two-iron : two-sulfur ferredoxin, and an iron-sulfur protein (Fig. 7). The ring hydroxylating enzyme which has been studied most extensively is naphthalene dioxygenase (NDO) produced by bacteria in the genus *Pseudomonas*. NDO is a multicomponent enzyme system containing three proteins: a flavoprotein (ferredoxin$_{NAP}$ reductase), a ferredoxin (ferredoxin$_{NAP}$), and a terminal oxidase (ISP$_{NAP}$) (Ensley *et al.* 1982, Ensley and Gibson 1983, Haigler and Gibson 1990a,b). This enzyme catalyzes the incorporation of two atoms of oxygen to form naphthalene *cis*-1,2-dihydrodiol. Recently Resnick *et al.* (1996) have reviewed literature relating to NDO and made some interesting observations. NDO is shown to have a relaxed substrate specificity and catalyzes the dioxygenation of many related 2- and 3-ring aromatic and hydroaromatic (benzocyclic) compounds to their respective *cis*-diols. In addition to *cis*-hydroxylation, NDO also catalyzes a variety of oxidations including monohydroxylation, desaturation (dehydrogenation), *O*- and *N*-dealkylation and sulfoxidation reactions of benzocyclic and alkyl-substituted aromatic compounds.

Recently researchers have investigated the discrete metabolic specificity of biphenyl dioxygenase and toluene dioxygenase from *Pseudomonas* spp. (Hirose *et al.* 1994,

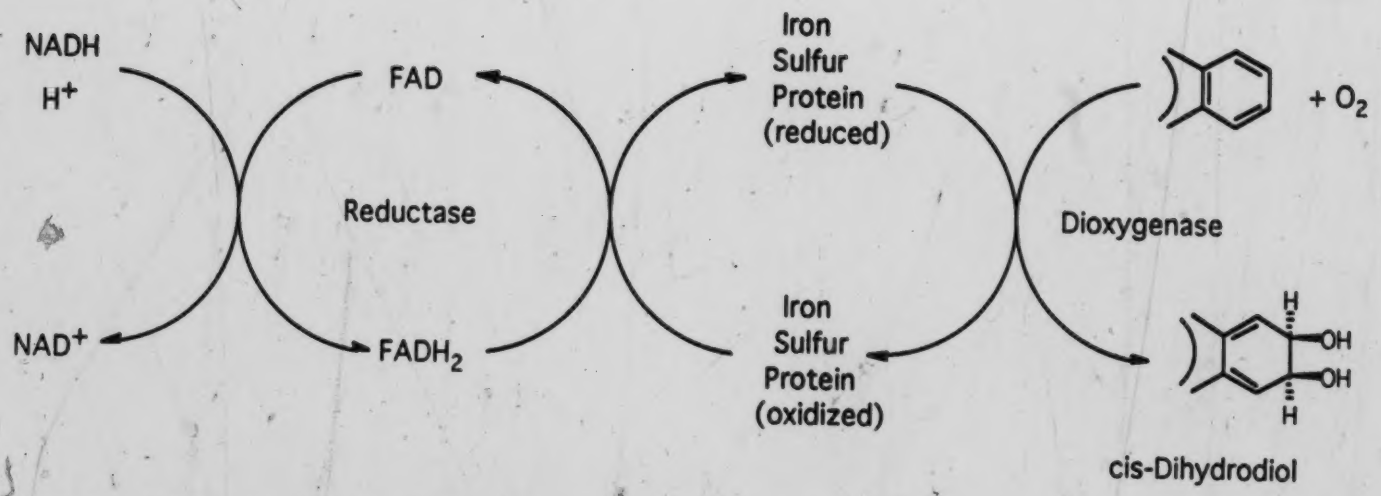


Fig. 7 Bacterial dioxygenase multicomponent enzyme system (after Cerniglia 1984).

Suyama *et al.* 1996). Both enzymes are multicomponent enzymes comprising four proteins: large and small subunits of the terminal dioxygenase, a ferredoxin, and a ferredoxin reductase. Despite the similarities of their amino acid sequences, the biphenyl-utilizing strain does not grow on toluene, and the toluene-utilizing strain does not grow on biphenyl. The inability of the biphenyl-utilizing strain to grow on toluene was due to a lack of initial oxidation of toluene by biphenyl dioxygenase (Furukawa *et al* 1993). It was found that the large subunit of the terminal dioxygenase was responsible for the specificity (Suyama *et al.* 1996). This was shown when the large subunit of terminal biphenyl dioxygenase was replaced with the large subunit of terminal toluene dioxygenase by molecular cloning, and the biphenyl-utilizing strain could then utilize toluene for growth.

2.5.2 Cytochrome P450 monooxygenases.

Cytochrome P450 is a well known oxidative enzyme present in numerous organisms ranging from bacteria to fungi and mammals. It plays an important role in the biosynthesis of sterols and in the metabolism of hydrocarbons, fatty acids, and xenobiotic compounds (Cerniglia *et al.* 1992). This enzyme was first discovered in mammalian liver microsomal samples and described as a complex mixture of terminal oxidase (Omura and Sato 1964). This enzyme is named by its characteristic spectral absorption peak at 450 nm. This characteristic peak is observed in the difference spectrum obtained from the ferrous (reduced minus oxidized) form of this iron porphyrin enzyme, in the presence of carbon monoxide (Wiseman 1980). Cytochromes P450 have been described as "the most versatile biological catalysts known" (Munro and Lindsay 1996). They usually function as components of monooxygenases which are involved in the oxidation of a great variety of aliphatic and aromatic compounds (Cerniglia *et al.* 1992).

Cytochrome P450 monooxygenases are multi-component enzymatic systems in which the terminal oxygenase component is heme-containing (Poulos *et al.* 1986). They

are generally composed of three protein components: an NAD(P)H-dependent flavoprotein reductase that transfers reducing equivalents via an intermediary iron-sulfur protein (ferredoxin) to the terminal P450 component where, in the presence of molecular oxygen, the substrate is oxidized (Trower *et al.* 1988). Cytochromes P450 present in yeasts and filamentous fungi have been reviewed by Cerniglia *et al.* (1992). A strain of *Cunninghamella bainieri* reportedly had a broad specificity cytochrome P450 that can catalyze the *N*-demethylation of 4-dimethylaminoantipyrine, the *O*-demethylation of anisole and *p*-nitroanisole, and the hydroxylation of aniline, anisole, and naphthalene (Ferris *et al.* 1973, 1976). The aryl hydrocarbon hydroxylase of this fungal strain can be reconstituted by combining NADPH and NADPH-cytochrome c reductase with the purified cytochrome P450. The enzyme is inhibited by proadifen and metyrapone (Ferris *et al* 1976). The inhibition occurs by the interaction of inhibitors with the haem of P450 cytochromes which then inhibits the action of the enzyme (Coulson *et al.* 1984).

There have been numerous papers, dealing mainly with fungi, which describe the oxidation of various PAHs through the action of cytochrome P450 such as the hydroxylation of benzo[a]pyrene by *Saccharomyces cerevisiae* (Wiseman and Woods 1979), by *Cunninghamella elegans* (Cerniglia and Gibson 1980), by *Chrysosporium pannorum, Neurospora crassa, Penicillium sp.*, and *Trichoderma viridae* ; the hydroxylation of pyrene by *Crinipellis stipitaria* (Lange *et al.* 1994); the oxidation of phenanthrene by *Cunninghamella elegans, Syncephalastrum raceosum,* and *Phanerochaete chrysosporium* (Sutherland *et al.* 1993), by *Pleurotus ostreatus* (Bezalel *et al.* 1996a); the oxidation of anthracene, pyrene, fluorene, and dibenzothiophene by *P. ostreatus* (Bezalel *et al.* 1996b).

Thus nonlignolytic fungi metabolize PAHs by cytochrome P450 monooxygenases to form the corresponding dihydrodiols in the *trans*-configurations, or to phenols and their conjugates (Cerniglia *et al.* 1992). However, lignolytic fungi growing under lignolytic

conditions are able to cleave the aromatic rings and mineralize PAHs using ligninases as described earlier whereby the PAHs are initially oxidized to quinones and then to the ring cleavage products. On the other hand, under nonlignolytic conditions, lignolytic fungi metabolize PAHs to the corresponding *trans*-dihydrodiols, or to phenols and their conjugates (Bezalel *et al.* 1996b).

As well as in fungi, there have been some reports of actinomycete and cyanobacterial metabolism of PAHs by cytochrome P450 monooxygenases. *Streptomyces flavovirens* when grown on tryptone yeast extract broth utilized cytochrome P450 monooxygenase to catalyze the oxidation of phenanthrene to form a *trans*-dihydrodiol (Sutherland *et al.* 1990). The marine cyanobacterium *Agmenellum quadruplicatum* also metablized phenanthrene to phenanthrene *trans*-9,10-dihydrodiol (Narro *et al.* 1992). *Mycobacterium* sp. reportedly metabolized pyrene by both dioxygenase and monooxygenase enzymes to form *cis*- and *trans*-4,5-dihydrodiol respectively (Heitkamp *et al.* 1988). However, even though PAHs can be metabolized by cytochrome P450 from some bacteria, from literature reviews it has been shown clearly that this type of metabolism is mainly performed by fungi.

2.6 Genetic control of PAH metabolism.

In PAH metabolism by bacteria, the genetic control of naphthalene utilization as the sole source of carbon and energy is the most well studied (Yen and Serdar 1988). The genes encoding enzymes of naphthalene degradation are located on self-transmissible NAH plasmids in most bacteria. The NAH7 plasmid found in *Pseudomonas putida* strain G7 and G1 is one of the most investigated plasmids. This plasmid has two operons: a *nah* operon that encodes an upper metabolic pathway, for the metabolism of naphthalene to salicylate, and a *sal* operon that encodes a lower pathway for the metabolism of salicylate via catechol to pyruvate and acetaldehyde (Yen and Gunsalus 1982, Yen and Serdar 1988).

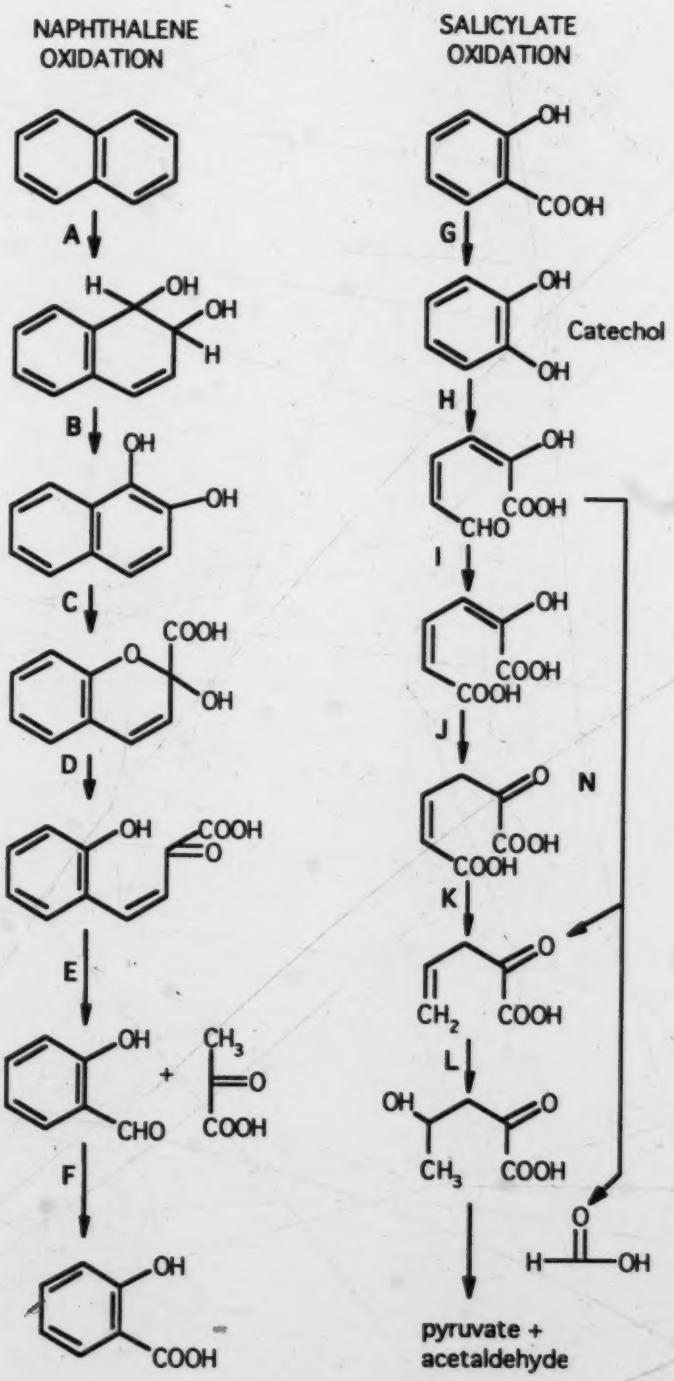
The NAH7 genes encoding enzymes of naphthalene degradation are shown in Fig. 8. The *nah* and *sal* operons are controlled by a positive regulatory gene, *nah*R, which is located immediately upstream of the *nahG* gene (Fig. 9). The activation of the *nah* operon needs both an inducer, such as salicylate, and the product of the regulatory gene *nahR* (Yen and Gunsalus 1982, 1985). The substrate, naphthalene, has no role in the operon induction. Salicylate also serves as an inducer for the *sal* operon enzymes. Recently, there has been evidence that NAH7 and similar plasmids are also involved in the degradation of PAHs other than naphthalene, that is anthracene and phenanthrene (Sanseverino *et al.* 1993, Menn *et al.* 1993). This finding indicates that the *nah* system of the NAH7 plasmid may mediate the degradation of more PAHs than previously thought which may play an important role in PAH degradation in contaminated soil.

2.7 Cometabolism.

Cometabolism is a term that has been in use for a long time. It has since been defined by Alexander (1967) to describe the transformation of an organic compound by a microorganism that was unable to use the substrate as a source of energy or as one of its constituent elements. Organisms do not obtain nutritional benefit from the substrate they cometabolize. The phenomenon occurs when a compound is transformed by a microorganism, but the organism is unable to grow on the compound and does not derive energy, carbon, or any other nutrients from the transformation reaction: the organism requires another substrate for growth. Therefore, cometabolism normally causes only modification of the compounds. If the compound is a PAH, this may result in either a decrease or an increase in toxicity. Further breakdown can be obtained through the combined action of different organisms (Alexander 1994).

Many microorganisms are capable of degrading PAHs cometabolically. *Mycobacterium* sp. have been shown to cometabolize pyrene (Heitkamp *et al.* 1988),

Fig. 8 Naphthalene and salicylate catabolic pathways in *Pseudomonas* sp. encoded by genes on the NAH7 plasmid. *Nah*A, naphthalene dioxygenase; *nahB*, *cis*-naphthalene dihydrodiol dehydrogenase; *nahC*, 1,2-dihydroxynaphthalene oxygenase; *nahD*, 2-hydroxychromene-2-carboxylate isomerase; *nahE*, 2-hydroxybenzalpyruvate aldolase; *nahF*, salicyladehyde dehydrogenase; *nahG*, salicylate hydroxylase; *nahH*, catechol oxygenase; *nahI*, 2-hydroxymuconic semialdehyde dehydrogenase; *nahJ*, 2-hydroxymuconate tautomerase; *nahK*, 4-oxalocrotonate decarboxylase; *nahL*, 2-oxo-4-pentenoate hydratase; *nahN*, 2-hydroxymuconic semialdehyde hydrolase (after Yen and Serdar 1988).


NAPHTHALENE
OXIDATION
SALICYLATE
OXIDATION
A
B
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D
E
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H
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J
K
L
N
Catechol
pyruvate +
acetaldehyde

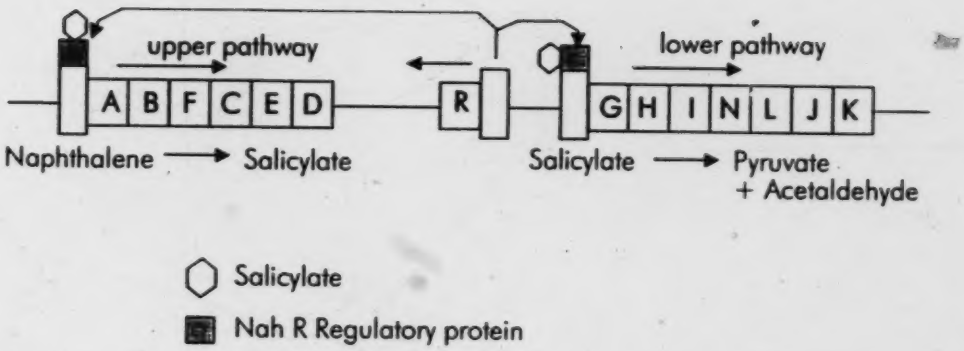


Fig. 9 Naphthalene catabolic genes organization and regulation (after Sutherland *et al.* 1995).

fluoranthene (Kelly *et al.* 1991), fluorene (Boldrin *et al.* 1993), pyrene, benz[a]anthracene, and benzo[a]pyrene] (Schneider *et al.* 1996). Sutherland *et al.* (1990) have shown that *Streptomyces flavovirens* metabolized phenanthrene to phenanthrene *trans*-9,10-dihydrodiol as the major product and a small amount of phenanthrol when the organism was grown on tryptone yeast extract broth. Jamison *et al.* (1971) reported that a strain of *Nocardia corallina* could metabolize tri- and tetramethylbenzenes, biphenyl, tetralin, and dimethylnaphthalene to yield a diversity of products. Fungi, which generally do not utilize PAHs as the sole carbon and energy source, also are able to metabolize several kinds of PAH when they are grown on other nutrients (Bezelel *et al.* 1996, Sutherland *et al.* 1991 and 1993, Cerniglia *et al.* 1989 and 1994, Pothuluri *et al.* 1993 and 1995).

The reactions employed in cometabolic conversions include hydroxylations, oxidations, denitrations, deaminations, hydrolyses, acylations, and cleavage of ether linkages (Schmitt *et al.* 1992). The cometabolism of some compounds is possible due to the fact that many enzymes are not absolutely specific and may act on more than one substrate including ones that are not typical cellular intermediates. Once the cosubstrate is metabolized it is not further transformed by other enzymes in the microorganism resulting in accumulation of that product. Cometabolism has some important roles in environments. Transformation of toxic parent compounds by cometabolism may result in the accumulation of dead-end products which tend to persist in the environment. However, the dead-end products produced from one organism in nature may be the substrates for the other organisms and may eventually be degraded completely (Alexander 1994).

2.8 Research objectives and rationale.

The mixed bacterial culture obtained from creosote-contaminated soil that was selected for growth on anthracene as sole carbon and energy source was comprised of two groups of bacteria based on their growth rates. The fast growing isolates were identified as

Gram negative *Pseudomonas* spp. (Gray *et al.* 1994) but little was known about the slow growing isolates except that they were Gram positive, acid-fast bacteria. Preliminary studies on the slow growing isolates revealed that of a number of PAH compounds tested, the isolates could grow only on anthracene indicating that they had a narrow substrate specificity for growth on PAHs, although they were able to grow on many complex media. This suggested that they had an initial dioxygenase of unusually narrow specificity or that these organisms used another metabolic pathway for growth on anthracene.

Many *Pseudomonas* spp. able to grow on anthracene can usually use naphthalene and phenanthrene as sole carbon and energy source for growth indicating a relaxed substrate specificity. Anthracene degradation by *Pseudomonas* spp. has been extensive studied and a pathway for its degradation proposed. In view of the difference in substrate specificity and because little was known about the metabolism of anthracene other than by Gram negative bacteria and fungi, I decided to study the slow growing, anthracene-utilizing bacteria.

The research objectives of this thesis were:

1. To isolate, purify, and characterize the slow-growing, anthracene-utilizing bacteria and study their growth conditions.
2. To study the pathway of anthracene metabolism by the slow-growing isolates.
3. To investigate the substrate specificity of the oxygenase enzyme involved in the initial ring hydroxylation of PAHs.

3. MATERIALS AND METHODS.

3.1 Culture methods.

3.1.1 Growth media and incubation conditions.

Anthracene-degrading bacteria were grown in one of the following complex media: Brain Heart Infusion (BHI, Difco), Trypticase Soy Broth (TSB, Baltimore Biological Laboratories), Nutrient Broth (NB, Difco). Alternatively, cultures were grown in a mineral salts (MS) medium which contained (g/l): KH_2PO_4, 1.6; K_2HPO_4, 2.4; NH_4Cl, 1.0; Na_2SO_4, 2.0; KNO_3, 2.0; $FeSO_4.7H_2O$, 0.007; and 1.0 ml of the trace metals solution (g/l of $CaCl_2.2H_2O$,3.7; H_3BO_3,2.5; $MnCl_2$,0.87; $FeCl_3$,0.65; $ZnCl_2$,0.44; $Na_2MoO_4.2H_2O$,0.29; $CoCl_2$,0.01; and $CuCl_2$,0.0001) (Fedorak and Grbic'-Galic' 1991). The organic compounds used as the carbon sources were added to MS medium at 0.05 % (w/v) unless stated otherwise. Anthracene and other PAH compounds were usually added directly to culture medium in their crystalline forms. Two-hundred-ml volumes of the culture medium in 500 ml Erlenmeyer flasks or 1 L volumes in 2 L flasks were incubated on a New Brunswick Gyrotary shaker at 200 rpm. To obtain the maximum sustained growth rate and cell yield, a coil (1.2 cm diameter) of stainless steel wire was placed at the bottom of the flask. Growth experiments were performed in the dark at 27°C. Growth in liquid cultures was determined by measuring optical density at 600 nm (OD_{600}) of the supernatant after the flask stood for 1 min to allow unused PAH crystals to settle to the bottom. Solid media were prepared by including 1.5% Bacto-agar (Difco) in the liquid media.

3.1.2 Isolation of slow-growing anthracene-degrading bacteria.

An anthracene-utilizing population maintained in our laboratory was used as the source for isolating of pure cultures of anthracene-degrading bacteria. Creosote-contaminated soil was used as the inoculum for the enrichment. Soil (7 g) was added to 200 ml of anthracene-MS medium in a 500 ml Erlenmeyer flask and the flask was incubated at 27°C with shaking. The mixed culture was enriched and maintained by monthly transfer of 10% (v/v) of settled culture fluid to fresh anthracene-MS medium (Gray *et al.* 1994). After six months a portion of this enrichment culture was serially diluted and spread on Plate Count Agar (PCA, Difco) plates. Plates were examined over a 2-week incubation period. They revealed two bacterial populations differentiated by growth rate, fast- and slow-growing, which were counted after 2 and 7 days of incubation respectively. The slow growing bacteria were purified by streaking on PCA plates and the process was repeated twice. Anthracene degradation by the pure isolates was examined after streaking MS agar plates and spraying with a solution of anthracene (20 mg) in dichloromethane (5 ml) (Kiyohara *et al.* 1982). Colonies surrounded by clear zones after 14 days incubation were selected for further study. Three representative strains, based on different colonial morphology, were chosen for further study and designated as 11S, S1, and 2F1. Purification and enumeration of the isolates were performed on PCA plates using the plate count method and examined after 1 week of incubation.

3.1.3 Maintenance culture and bacterial inocula.

Routine culture of anthracene-utilizing bacteria was carried out by monthly transfer of 10% (v/v) of settled culture fluid to fresh anthracene MS medium. Inocula were obtained either from colonies on PCA plates that were streaked from anthracene-MS medium-grown cultures of anthracene degrading bacteria, or directly from anthracene-MS medium-grown liquid cultures. In the latter case, the cell pellets were washed twice with

sterile MS medium and filtered through sterile glass wool to remove the remaining anthracene prior to being used as an inoculum.

3.2 Biochemical, chemical, and physiological characteristics of isolates 11S, S1, and 2F1.

3.2.1 Biochemical and physiological characterization.

General biochemical and physiological characterization tests were performed using methods described by Barrow and Feltham (1993) and Roberts *et al.* (1991).

3.2.2 Lysozyme sensitivity.

Lysozyme sensitivity tests were performed according to the methods described by Mordarska *et al.* (1978). Cells were grown in BHI broth, washed and resuspended in sterile distilled water to an OD_{600} of approximately 0.2 to 0.25. This cell suspension (7 ml) was treated with 0.4 ml of 1% (w/v) aqueous lysozyme and incubated at 37°C for 3 h, followed by addition of 0.7 ml of 20% (w/v) aqueous sodium dodecyl sulfate (SDS) prior to optical density measurement.

3.2.3 G+C content of DNA.

Mole percent guanine plus cytosine (mol% G+C) of DNA was determined by midpoint determination of the thermal melting (denaturation) profiles (Johnson 1981). *Escherichia coli* B was used as a reference strain.

3.2.3.1 Cell disruption for nucleic acid isolation.

Exponential phase cells of the three isolates (11S, S1, and 2F1) and *E. coli* B, grown in BHI broth, were harvested and resuspended in a volume of saline-EDTA buffer

(0.15 M NaCl, 0.01 M EDTA, pH 8.0) equal to 2.5% to 5% of the original culture volume. SDS was added to 1% and incubated in a 50°C water bath until cells were lysed, indicated by a rapid increase in viscosity and a change in the cell suspension from turbid to opalescent.

For acid-fast bacteria, cell pellets were resuspended in a 1:5 dilution of saline-EDTA buffer and lysozyme was added (4 mg/ml) to enhance lysis at 37°C before SDS addition.

3.2.3.2 DNA isolation.

The Marmur method (Marmur 1961) was employed to isolate bacterial DNA. The lysed cells were placed in a ground glass-stoppered flask and 5 M sodium perchlorate was added to a final concentration of 1 M followed by 0.5 volume of chloroform-isopentanol (24:1, v/v). The flask was shaken on a wrist-action shaker at low speed for 30 min. The emulsion was centrifuged at 17,000 x g and 4°C for 10 min. The upper aqueous layer was carefully withdrawn by pipet and the chloroform-isopentanol extraction repeated.

The aqueous phase was then slowly overlaid with 2 volumes of cold 95% ethanol in a beaker. The precipitated DNA was collected with a glass stirring rod by gently stirring the two phases while spinning the rod. The rod was drained vertically for a few minutes to remove the remaining ethanol. The "spooled" DNA was placed in 0.1x saline sodium citrate buffer (SSC; 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) until it dissolved completely. The SSC buffer concentration was adjusted to 1x followed by addition of RNase to 50 µg/ml. After incubation at 37°C for 30 min, the DNA preparation was again extracted with chloroform-isopentanol to remove protein.

The DNA so obtained was precipitated with ethanol by "spooling" and dissolved in 0.1x SSC buffer. This process was repeated 3 times to remove ribonucleotides. The DNA

was finally dissolved in 0.1x SSC buffer and stored at -20°C for thermal melting point (*Tm*) determination.

3.2.3.3 Determination of DNA thermal melting point.

SSC buffer was used both at the recommended half-strength (0.5x, Johnson 1981) and at 0.05x for a possibly lower *Tm* (personal communication, J.M. Foght).

DNA preparations of the three isolates and *E. coli* B (reference DNA) were dialyzed in the same batch of SSC buffer overnight to assure that the isolates and reference DNA were at the same ionic strength. The melting profiles of DNA were determined using a Philips Unicam PU 8740 automatic recording spectrophotometer with an electronically heated cuvette holder. In 0.5x SSC buffer, the temperature started at 70°C and 80°C for *E. coli* and the isolates respectively whereas in 0.05x SSC buffer, 55°C was used for *E. coli* and 65°C for the isolates. The temperature was increased linearly at 2°C per min over the entire range. *Tm* was determined from a recording graph of absorbance vs temperature. Mol% G+C of the isolates were calculated using the following equation:

$$\text{mol\% G+C} = \text{mol\% G+C}_{\text{ref}} + 1.99\,[\,Tm\,(\text{x}) - Tm\,(\text{ref})\,]$$

where mol% $G+C_{ref}$ is the known mole percent G+C of *E. coli* B and $Tm(x)$ and $Tm(ref)$ are the *Tm* values determined for DNA of the isolates and *E. coli* B, respectively, under the same experimental conditions (Johnson 1981).

3.2.4 Qualitative evaluation of mycolic acids.

3.2.4.1 Whole cell methanolysate.

Whole cell methanolysate analyses were performed using the method described by Minnikin *et al.* (1975) using bacteria grown in TSB. *Corynebacterium glutamicum* was grown at 37°C for 2 days. All other incubations were at 27°C for 5 days except for

Nocardia spp. which were incubated for 13 days. The organisms were then killed by shaking with 1% formaldehyde, harvested by centrifugation, washed with distilled water and freeze-dried. Dried cells were degraded using acid methanolysis to obtain crude mycolic acid methyl esters. Dried cells (~50 mg) were placed in a 20 ml test tube closed with a polytetrafluoroethylene-lined screw cap. A mixture (3 ml) of dry methanol-toluene-sulfuric acid (30:15:1) was added, mixed thoroughly by vortex for 2 min and the tightly closed tube was incubated at 75°C in a heating block for ~18 h. After cooling to room temperature, the mixture was extracted with 2 ml of light petroleum ether (b.p. 60-80°C). The methanolysate was then concentrated under nitrogen to 100-200 µl prior to thin layer chromatographic (TLC) analysis.

3.2.4.2 Thin layer chromatography.

Methanolysate extracts (5-50 µl) were applied to silica gel sheets (Kodak Chromogram 13181 with fluorescent indicator) and developed twice in light petroleum : diethyl ether (85:15, v/v). The separated components were visualized by spraying with a solution of molybdophosphoric acid (1 g) in ethanol (10 ml) followed by heating at 120°C for 15 min. Components appeared as dark blue-green spots on a white background.

3.3 Scanning electron microscopy.

Anthracene-degrading isolate S1 always appeared aggregated when grown on anthracene-MS medium in a shake flask. Upon removal from the shaker, the aggregates settled at the bottom of the flask and were examined by phase-contrast microscopy. Observations revealed cells aggregated and attached to anthracene crystals. To confirm this phenomenon, the culture samples were prepared for examination by scanning electron microscopy.

Isolate S1 was grown in anthracene-MS medium in an Erlenmeyer flask for 3 weeks. The culture was swirled for 10 seconds and allowed to sit for 1 min before decanting the cell suspension from the residual anthracene crystals . This provided two kinds of samples: bacterial cells suspended in the liquid culture and the sediment of anthracene that settled to the bottom of the culture flask.

The cell suspension was centrifuged at 10,000 x g for 10 min. Cell pellets were washed twice with and resuspended in a volume of Millonig's buffer (Millonig 1961) (16.8 g $NaH_2PO_4.H_2O$; 3.86 g NaOH; 5.4 g glucose; 0.05 g $CaCl_2$ and 1 L distilled water, pH 7.2) equal to about 5% of the original volume before being attached to a poly-L-lysine layer coated on glass coverslips prior to fixation stages.

The poly-L-lysine layer was prepared using the method described by Sanders *et al.* (1975). A 25 µl drop of poly-L-lysine hydrobromide (M.W. 132,300, Sigma Chemical Co.; 1 mg/ml solution in 10 mM phosphate buffer pH 7.2 - 0.15 M NaCl) was placed on one side of 1.25 cm diameter coverslip. The coated coverslips were kept at room temperature for 1 h before rinsing in distilled water and then placed in a Petri dish in a moist chamber. The coverslips were partially blotted with filter paper. Then 20 µl of cell suspension was placed on the coated coverslips which were kept at 4°C in a moist chamber for 14 h for cell settling. Cells which attached to the lysine layer were then fixed with glutaraldehyde and osmium tetroxide.

The sediment of anthracene left after decanting the cell suspension was washed twice and resuspended in Millonig's buffer in 13 x 100 mm test tubes and fixed with glutaraldehyde and osmium tetroxide.

Fixation of the samples in test tubes and on poly-L-lysine coated coverslips was carried out at 4°C. They were prefixed with 3% (v/v) glutaraldehyde (grade 1, Sigma Chemical Co.) in Millonig's buffer for 3 h and washed in the same buffer for 1 h (buffer

changed at 30 min). The samples were post fixed in buffered 2% (w/v) osmium tetroxide for 2 h and rinsed briefly in distilled water.

The fixed samples were then dehydrated sequentially in a series of ethanol solutions (% concentration in water: 30, 50, 70, 80, and 90) for 15 min each, followed by four 10 min incubations in absolute ethanol. The anthracene sediment from test tubes was placed on adhesive tape and adhered to cover slips. The dehydrated samples on coverslips were then critical point dried and sputtered with gold. Scanning electron micrographs were taken by G. Braybrook in the Department of Geology, University of Alberta using a Cambridge Stereoscan 250 scanning electron microscope at 10-20 kV.

3.4 PAH mineralization by induced cells.

Mineralization experiments were carried out using [U-^{14}C]naphthalene, [9-^{14}C]phenanthrene, and [9-^{14}C]anthracene. Since none of the 3 isolates could grow on naphthalene or phenanthrene, starter cultures were all grown in anthracene-MS medium. Cultures were harvested, washed twice, and resuspended in MS medium to an OD_{600} of about 10 as a stock inoculum. The stock inoculum was then diluted 100-fold to give final cell concentrations of about 10^8 CFU/ml (OD_{600} of approximately 0.1) in a 500 ml flask, equipped with side arms for sampling, that contained 200 ml of MS medium plus 1 mg PAH and approximately 300,000 dpm of [^{14}C]PAH added as a dichloromethane solution. The flasks were shaken in the dark at 200 rpm for 96 h, and cell numbers were determined at the beginning and at the end of the incubation. Sterile controls of each PAH were included to detect abiotic degradation and volatilization of the PAH. A positive mineralization control for each PAH was performed using *Pseudomonas* sp. strain HLA, an anthracene-utilizing bacterium (Foght and Westlake 1991). The evolution of $^{14}CO_2$ from radiolabelled anthracene, phenanthrene and naphthalene was measured by the method of Fedorak *et al.* (1982). Samples of liquid (5 ml) and headspace gas (10 ml) were

withdrawn by syringe and replaced by 15 ml sterile air. The liquid and gas were injected into a pre-evacuated 38-ml serum bottle, the liquid acidified with 4 N H_2SO_4 and the evolved CO_2 flushed with N_2 through two scintillation vials in series. Each vial contained 10 ml ACS fluor and 1 ml Carbo Sorb II for trapping CO_2. The vials were then analyzed by liquid scintillation spectrometry (Beckman LS 3801). The amount of radiolabel recovered as $^{14}CO_2$ was reported as percentage of the amount of radiolabel added originally.

3.5 Production and recovery of metabolites from anthracene-degrading isolate S1.

Preliminary studies of anthracene metabolites from isolates 11S, S1, and 2F1 were performed by organic solvent extraction of their spent media. The isolates were grown on anthracene-MS medium for 1-6 weeks in 200 ml volume as a preliminary small scale experiment. Since all three strains yielded similar metabolites shown by TLC and gas chromatography (GC), only strain S1 was later chosen for the metabolite study, based on its fastest growth rate.

The spent medium was centrifuged at 10,000 x g for 10 min at 4°C to remove the cells and unused anthracene crystals. The supernatant was then filtered through a 0.45 μm membrane filter (Millipore Corporation, Bedford, MA) to remove fine anthracene crystals. This supernatant was extracted at neutral pH in a separatory funnel three times with 0.25 volume of dichloromethane (neutral extract). Then, the supernatant was adjusted to pH < 2 with 4 N H_2SO_4 and extracted 3 times with 0.25 volumes of ethyl acetate (acid extract). The organic layers were dried over anhydrous sodium sulfate and concentrated by rotary evaporation. The extracts were subsequently concentrated under a gentle stream of nitrogen to a 200 μl volume prior to TLC and GC analyses. In larger batch culture of the

S1 isolate (6x1 L volume of spent medium), the final extract was concentrated to 1.2 ml prior to TLC and GC analyses.

3.6 Production and recovery of metabolites from *Pseudomonas* sp. strain HL4.

Pseudomonas sp. strain HL4 (Foght and Westlake 1991) was included in the metabolite study of the isolates 11S, S1, and 2F1 for comparison purposes. The bacterium was grown in 200 ml anthracene-MS medium. The spent medium at varied incubation time from 1-6 weeks was extracted with organic solvent at the neutral and acidic pH to yield the neutral and acid extracts as previously described in section 3.5.

3.7 Production and isolation of anthracene and phenanthrene dihydrodiols from *Pseudomonas fluorescens* LP6a (pLP6a :: *Tn* 5) mutant D1.

P. fluorescens LP6a (pLP6a :: *Tn*5) mutant D1 (Foght and Westlake 1996), a transposon mutant of the wild type strain LP6a was employed to produce *cis*-1,2-dihydroxy-1,2-dihydroanthracene (anthracene *cis*-1,2-dihydrodiol) from anthracene, *cis*-1,2-dihydroxy-1,2-dihydrophenanthrene (phenanthrene *cis*-1,2-dihydrodiol) and *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene (phenanthrene *cis*-3,4-dihydrodiol) from phenanthrene to be used for comparison purposes.

P. fluorescens LP6a mutant D1 has a mutation in the *nah*B gene which encodes *cis*-1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase resulting in accumulation of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (naphthalene *cis*-1,2-dihydrodiol) (Foght and Westlake 1996). Thus, after induction by salicylic acid, this strain oxidizes anthracene and phenanthrene to their respective dihydrodiols which accumulate in the supernatant.

Media to grow pLP6a :: *Tn*5 mutants contained kanamycin (50 µg/ml) to ensure maintenance of transposon and plasmid. A bacterial colony from a trypticase soy agar plate

was inoculated into a flask containing trypticase soy broth (TSB) and incubated on a shaker at 27°C overnight. An 25 ml-aliquot was then transferred to each of 6 flasks (2 L) containing 1 L TSB. The incubation was continued for another day before salicylic acid was added to 0.5 mM final concentration. The culture was shaken for a minimum of 3 h prior to harvesting by centrifugation. Cell pellets were resuspended in 6 x 1 L of 3 mM phosphate buffer, pH 7.2 followed by addition of either anthracene or phenanthrene (dissolved in dimethylformamide, DMF) to 2.5 mM final concentration and incubation overnight. Supernatant was obtained from centrifugation and extracted at neutral pH with 3 x 0.25 vol dichloromethane (DCM). The extract was dried over anhydrous sodium sulfate and concentrated to 10 ml by rotary evaporation and under N_2 stream. The extract was stored at -20°C.

3.8 Production of phenols from chemical dehydration of PAH dihydrodiols.

Dehydration of dihydrodiols was performed using the methods described by Jerina *et al.* (1976). Crude neutral extracts containing dihydrodiols (~1.8 ml) were evaporated under nitrogen to dryness and redissolved in 7 ml of 5 N HCl. This solution was heated in a sandbath at 100°C for 10 min under a stream of nitrogen, then cooled to room temperature before extraction three times with 3 ml DCM. The extract was dried over anhydrous sodium sulfate, concentrated to a 1-ml volume under nitrogen and stored at -20°C.

3.9 Analytical methods.

Neutral and acid extracts from cultures of the isolates 11S, S1, 2F1 and *Pseudomonas* sp. strain HL4 were examined by TLC. Analytical plastic sheets coated with silica gel (100 µm Kodak Chromagram #13181 with fluorescent indicator; Eastman Kodak

Company, N.Y.) were employed. Extracts (5-10 µl) were applied and the plates developed in benzene-acetone-acetic acid (85:15:5, v/v/v). The spots were visualized under UV before spraying with reagents. Phenolic compounds gave dark greenish blue colors after being sprayed with Gibbs' reagent (2,6-dichloroquinone-4-choroimide; Sigma) followed by 10% (w/v) Na_2CO_3. *o*-Dianisidine in acetic acid (saturated) was used to detect aldehydes and ketones. Acids were detected by spraying with bromocresol green solution. *o*-Phenylenediamine in trichloroacetic acid was used to detect α-ketoacids (Krebs *et al.* 1969).

Neutral and acid extracts from isolates 11S, S1, 2F1 and *Pseudomonas* sp. strain HL4 were analyzed by GC using a Hewlett Packard (HP) model 5730A gas chromatograph equipped with a flame ionization detector (FID). Injector and detector temperatures were set at 250°C. The DB-5 capillary column used (30 m long, 0.25 mm internal diameter, 0.25 µm film thickness) was held at 90°C for 2 min, increased by 4°C per min to 250°C, and then held for 16 min at this final temperature. An HP model 3390A integrator was used to record chromatograms.

Selected extracts were analyzed by electron impact and chemical ionization (using ammonia as the reagent gas) GC-mass spectrometry (GC-MS) at the Mass Spectrometry Laboratory in the Chemistry Department at the University of Alberta. GC-Fourier transform infrared spectroscopy (GC-FTIR) analysis was performed at the Spectral Services Laboratory in the Chemistry Department at the University of Alberta.

3.10 Derivatization methods.

In order to detect polar metabolites by GC, three methods of derivatization were used. Extracts were dissolved in DCM or ethyl acetate (EtAc) and derivatized with *N,O*-bis(trimethylsilyl)acetamide (BSA) (Pierce 1989). This reaction was performed by mixing equal volumes of the extract and BSA and shaking vigorously for 1 min before GC

analysis. In some cases, an ethereal solution of diazomethane was used to methylate samples. Diazomethane was prepared from 1-methyl-3-nitro-1-nitrosoguanidine using a millimole size generator according to manufacturer's instructions (Wheaton, Millville, NJ) and was added to the extracts immediately after preparation. Reacted samples were allowed to sit overnight before GC analysis. Derivatization of the extracts with butaneboronic acid (BTB) (Brooks and Maclean 1971) was performed in some samples in an attempt to detect *cis*-dihydrodiol metabolites. In this method, 1.0 ml butaneboronic acid solution (10 mg/ml in EtAc) was added to 1 ml of the extract dissolved in EtAc and the mixture incubated at 30°C for 5 min. Samples were cooled to room temperature before GC analysis.

3.11 Purification of the unknown metabolite obtained from the isolate S1 grown on anthracene.

3.11.1 High performance liquid chromatography (HPLC).

HPLC analyses were carried out on a WISP model 712 Automatic Injector and a Model 486 Tunable Absorbance Detector, (Waters Scientific Co., Mississauga). The integrator was an HP model 3392A. An analytical reverse phase column (spherisorb-10 RP-18, 100 x 4.6 mm, Brownlee Labs Inc., Santa Clara) was used for a preliminary study to find the separation conditions for preparative scale using a 250 x 10 mm PrepPak Cartridge (Delta-Pak C18, 15 µm, 100 Å, Waters, Milford, MA). The neutral crude extract in dichloromethane was evaporated to dryness under a gentle stream of nitrogen, redissolved in methanol and purified on the preparative column with an isocratic mobile phase of 40% methanol in water at a flow rate of 3 ml/min. The eluant was monitored at 254 nm. Under these conditions, the unknown compound which eluted with a retention time between 7.22 - 7.47 min was collected from repeated injections of the neutral crude

extract. Fractions were pooled and extracted with 3 x 0.25 vol DCM. The organic extract was then concentrated to a small volume by rotary evaporation. The concentration of the compound was estimated using GC by relative comparison of peak areas with those of 2,3-dihydroxynaphthalene after the samples were derivatized with BSA unless stated otherwise.

3.11.2 Silica gel column chromatography.

Attempts to purify anthracene dihydrodiol from *P. fluorescens* LP6a mutant D1 and the unknown metabolite from the isolate S1 were performed by silica gel column chromatography using the methods described by Fedorak and Andersson (1992) with some modifications. Silica gel (40-63 µm, General Intermediates of Canada) was activated at 125 °C overnight prior to suspending in a developing solvent (benzene-acetone-acetic acid; 90:8:2) for 24 h. The silica gel slurry was then packed into a glass column (30 cm x 1.2 cm) and the column was washed with 5 column volumes of the developing solvent before applying the sample. The neutral extract in DCM was evaporated to dryness under a stream of nitrogen and redissolved in the developing solvent before applying to the column. Fractions (1 ml each) were collected, concentrated under nitrogen and analyzed by TLC. Fractions that contained the unknown metabolite from the isolate S1 and phenanthrene dihydrodiol from pLP6a :: *Tn*5 mutant were pooled, concentrated, and analyzed by GC.

3.11.3 Preparative silica gel TLC.

Preparative TLC was carried out using glass plates precoated with a preparative layer (500 µm) of silica gel containing fluorescent indicator (254 nm) (20 x 20 cm, Si500F; J. T. Baker Inc., N.J.). A benzene-acetone-acetic acid (85:15:5, v/v/v) solvent system

was used. Bands which had strong fluorescence under short wavelength UV were scraped off and extracted with DCM.

3.12 Purification of acid dehydrated products of the unknown metabolite from isolate S1.

The dehydrated neutral extract of the isolate S1 in DCM (40 ml) was extracted 3 times with 15 ml, 5% NaOH solution. By this method, the acidic phenols were partitioned into the alkali and separated from non-acidic compounds in the organic solvent phase. The resultant alkali portion was acidified to pH 2 with 4 N H_2SO_4 and extracted 3 times with 0.25 volume of DCM. The DCM extract was dried over anhydrous sodium sulfate and concentrated under nitrogen to dryness. The dried extract was redissolved in methanol and subjected to further purification using a preparative HPLC column (Delta-Pak C18, 15 µm, 100 Å, Waters, Milford, MA) with an isocratic mobile phase of 30% acetonitrile in 5 mM phosphate buffer pH 3.4 at a flow rate of 4 ml/min. The effluent was monitored at 214 nm. Under these conditions, the dehydrated unknown compound which eluted with a retention time between 50 - 58 min was collected from repeated injections of the sample. Fractions were pooled and extracted with 3 x 0.25 vol DCM. The organic extract was then concentrated to a small volume by rotary evaporation. The purified phenolic compound was also analyzed by GC to estimate purity.

3.13 Chemical synthesis of standard anthrols.

3.13.1 Synthesis of 9-anthrol.

9-Anthrol was prepared from anthrone using the methods described by Meyer (1911). Anthrone was dissolved in 7.5 % boiling NaOH for 5 min and cooled quickly to -5°C. The cool solution was then poured into cold 5% H_2SO_4 which subsequently yielded

a yellow precipitate. The precipitate containing 9-anthrol was washed throughly with ice-water, filtered, and dried in a desiccator.

3.13.2 Synthesis of 1- and 2-anthrols.

Synthesis of 1- and 2-anthrols was from the corresponding 1- and 2-aminoanthracenes by decomposition of the diazonium salts as described by Cohen *et al.* (1977). Aminoanthracene (2 mM) was dissolved in 2 ml of hot 35% sulfuric acid and then cooled to below 15°C. Ice (2 g) was added followed by 0.18 g of sodium nitrite which had been dissolved in 2 ml of water at 4°C. The solution was stirred for 10 min before urea (~5 mg) was added. A solution of cupric nitrate trihydrate (7.5 g in 70 ml of water) was added, followed by cuprous oxide (0.26 g) and stirred for another 20 min. The mixture was then extracted with 3 x 0.25 vol diethyl ether. The ethereal solution was then back-extracted into 5% NaOH which was then acidified with 7 N H_2SO_4 to pH 2 before extraction with DCM. The DCM extract which contained anthrol was analyzed by GC. The anthrol standards were confirmed by GC-MS and GC-FTIR and not further purified.

3.14 Synthesis of *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene.

Trans-9,10-dihydroxy-9,10-dihydrophenanthrene (phenanthrene *trans*-9,10-dihydrodiol) was prepared using the methods described by Harvey *et al.* (1975). Phenanthrene-9,10-quinone (0.7 mM) was reacted in a Soxhlet apparatus with diethyl ether (200 ml) containing lithium aluminium hydride (5 mM) for 15 h under N_2. The reaction mixture was cooled to room temperature, and acetic acid solution was added. The ether extract was washed with sodium bicarbonate solution, extracted with EtAc, dried over anhydrous sodium sulfate, and concentrated by rotary evaporation. The presence of *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene in the extract was confirmed by GC-MS analyses.

3.15 Cometabolism of various PAHs by the isolate S1.

3.15.1 Anthracene as the growth substrate.

In order to examine if the isolate S1 was able to transform other potential substrate PAHs by cometabolism when grown on anthracene, the strain S1 was grown on anthracene-MS medium in the presence of one of the following PAHs: naphthalene, biphenyl, acenaphthene, 2-methylphenanthrene, fluorene, fluoranthene, pyrene, chrysene, benzanthracene, and naphthacene. Sterile controls of the individual PAH were included. All flasks were shaken for 4 weeks before proceeding to extraction. The neutral extracts were analyzed by GC-FID and GC-MS.

3.15.2 Glucose as the growth substrate.

To examine if isolate S1 was able to transform other potential substrate PAHs by cometabolism when grown on glucose, S1 was grown in a 1% glucose - MS medium in the presence of either naphthalene (0.025%) or chrysene (0.0125%). The inoculum for the glucose-MS medium was from anthracene-MS liquid culture and the cell pellet was washed with mineral salts medium to remove any anthracene crystals. All flasks were shaken for 10 days prior to extraction. The neutral extracts were analyzed by GC and compared to extracts of control flasks without naphthalene or chrysene.

3.16 Cometabolism of phenanthrene and chrysene by anthracene-grown *Rhodococcus* S1 in the presence of cytochrome P450 inhibitors.

To investigate whether the transformation products of PAH obtained from *Rhodococcus* S1 were the oxidation products formed by cytochrome P450

monooxygenase, cometabolism of phenanthrene and chrysene was examined in the presence of the cytochrome P450 inhibitors metyrapone and ancymidol.

Rhodococcus S1 from a maintenance culture was inoculated (10%) into 200 ml anthracene-MS medium in 500 ml Erlenmeyer flask and incubated for about 4 days to obtain a cell density (OD_{600}) of about 0.3 before addition of the cytochrome P450 inhibitors metyrapone (0.4 and 1.0 mM) or ancymidol (0.2 and 1.6 mM) in dimethylsulfoxide. The cultures with inhibitors were incubated for 30 min, and then phenanthrene (0.005%) and chrysene (0.0025%) were added in dimethylsulfoxide. The cultures were incubated for 10 more days before solvent extraction of the medium. A culture without the inhibitor was used as the control. Dibenzothiophene was added into each culture flask before extraction as the internal standard. The cultures were then extracted with DCM and analyzed by GC-FID for the formation of phenanthrene dihydrodiol and chrysene dihydrodiol.

4. RESULTS

4.1 Characterization of anthracene-degrading bacteria as strains of *Rhodococcus* spp.

Three isolates were chosen from among the slow growing, anthracene-degrading bacteria and designated as 11S, S1, and 2F1. They were all aerobic, nonmotile, nonsporulating, rod-shaped, Gram positive, and acid-fast. Colonies appeared on PCA after 5-6 days at 27°C. The colonies of 11S and 2F1 were smooth, shiny, creamy, and entire, whereas those of S1 were opaque, creamy, rough surfaced, and uneven edged, with a convex center. On MS agar plates coated with anthracene, the isolates appeared as pin-point colonies surrounded by clearing zones indicating the degradation of anthracene after 2 weeks of incubation. Most of the studies in this work were carried out on strain S1 because it had the fastest growth rate among the three isolates. The general biochemical, chemical, and physiological characteristics of the three isolates were similar and are given in Table 5.

Chemotaxonomic characterization of isolates 11S, S1, and 2F1 were performed. Several mycolic acid-containing bacteria were employed as the control organisms including hydrocarbon degrading strains of *Corynebacterium*, *Rhodococcus*, *Nocardia*, and *Mycobacterium*. The methanolysate patterns of the isolates and these reference strains are shown in Fig. 10. Of the reference strains tested, only *Mycobacterium* gave multispot patterns of mycolic acid methyl esters (R_f, 0.1-0.5; Minnikin *et al.* 1975) on a TLC plate, while *Corynebacterium*, *Rhodococcus*, *Nocardia*, and the unknowns all gave a single spot pattern. Taxonomic confirmation by pyrolysis gas chromatography was performed by Dr R. Coleman at Alberta Environmental Centre, Vegreville, Alberta. The results showed that methyl mycolates from the isolates 11S, S1, 2F1 yielded fatty acid esters with carbon chains that were shorter than 20:0 (C_{14}-C_{18}), a feature of the genera *Rhodococcus*,

Table 5 Biochemical, chemical, and physiological characteristics of slow-growing, anthracene-degrading, acid-fast bacteria.

Characteristics	Bacterial isolate		
	11S	S1	2F1
Catalase production	+	+	+
Nitrate reduction	-	-	-
Arylsulfatase	-	-	-
Acid phosphatase	+	+	+
Iron uptake	-	-	-
Colonies photochromogenic	-	-	-
Growth at:			
37°C	+	+	+
45°C	-	-	-
Acid production from:			
arabinose	-	-	-
dulcitol	-	-	-
xylose	-	-	-
Utilization of:			
citrate	-	-	-
mannitol	-	-	-
oxalate	-	-	-
Growth in the presence of:			
malachite green, 0.01%	+	+	+
pyronin B, 0.01%	+ (weak)	+	+
$NH_2OH.HCl$, 500 μg/ml	+	+	+
picrate, 0.02%	-	-	-
NaCl, 5.0%	+	-	+ (weak)
Growth on MacConkey medium without crystal violet	-	-	-
Lysozyme sensitivity	+	+	+
G+C content of DNA	65%	66%	67%

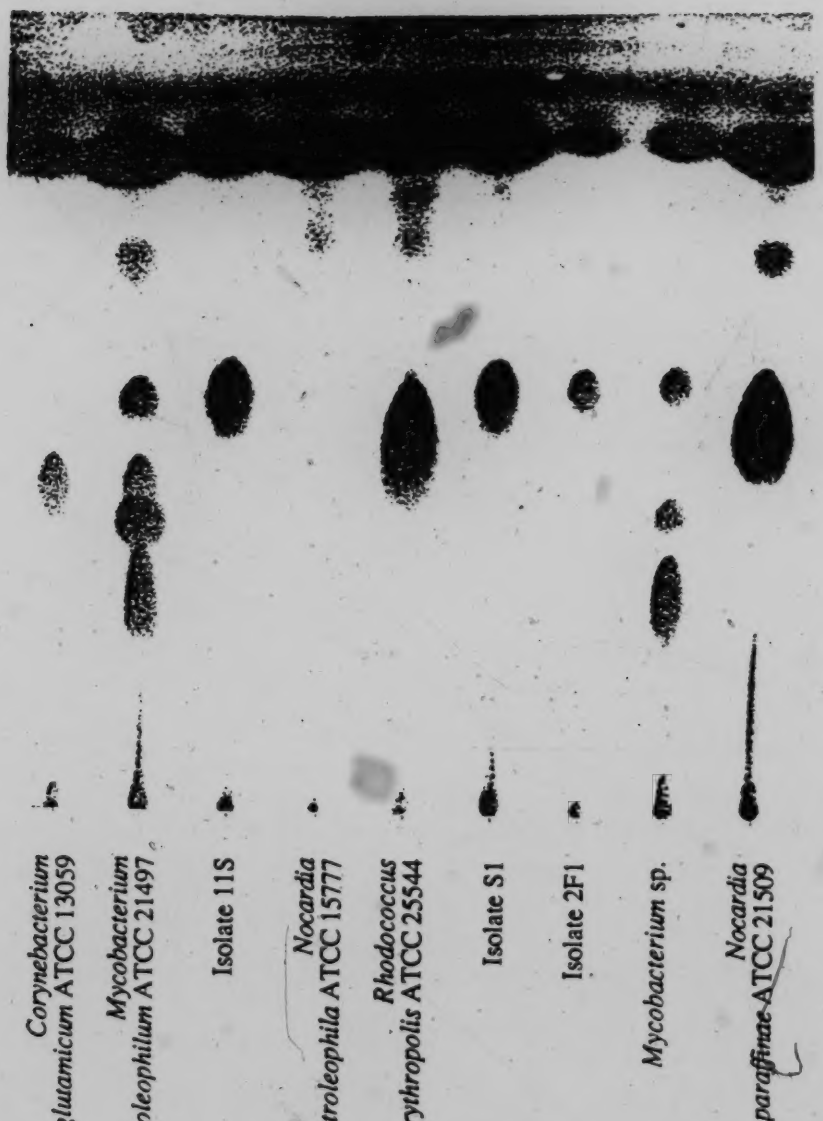


Fig. 10 Thin-layer chromatogram of the whole cell methanolysates of isolates 11S, S1, 2F1 and some mycolic acid containing bacteria.

Nocardia, and *Corynebacterium*. All three isolates had diaminopimelic acid as a major component of the cell wall peptidoglycan. The major fatty acids produced were 16:0, 18:1, 16:1, 14:0, and possibly tuberculostearic acid (10-methyl 18:0). On the basis of these characteristics all three isolates of anthracene-utilizing bacteria were identified as *Rhodococcus* spp. (Sneath *et al.* 1986; Goodfellow 1992).

4.2 Growth and metabolism of *Rhodococcus* strains.

4.2.1 Growth in complex and anthracene-MS media.

All three isolates were able to grow on standard laboratory media including PCA, BHI, TSB, and NB. When grown in complex media such as BHI, TSB, and NB, shown in Fig. 11, the three isolates had generation time of approximately 10-14 h whereas in anthracene-MS medium, Fig. 12, the generation time was approximately 1.4 - 3 days with a lag period of about 2 days. They all grew well at 27°C and 30°C, poorly at room temperature (22°C), and moderately at 37°C. No growth was observed at 45°C. All three isolates were able to adapt to growth on anthracene from these complex media, after two washes in MS medium to remove any complex medium components, with a lag period of about 2 days. When anthracene was supplied as fine amorphous crystals precipitated from *N, N'* -dimethylformamide, the increased surface area enabled more rapid growth (Fig. 13).

Growth of *Rhodococcus* S1 on anthracene crystals was revealed by scanning electron microscopy. The bacterial sediment and suspended cells of a 3-week culture were examined. It was found that when *Rhodococcus* S1 was growing in anthracene-MS medium, bacterial cells in the supernatant were found in two forms both as single cells (Fig. 14A) and as clumps (Fig. 14B). The size of a 3-week old cell varied from 0.5 x 1.2 µm to 0.4 x 2.8 µm. In addition, scanning electron micrograph also revealed the bacterial

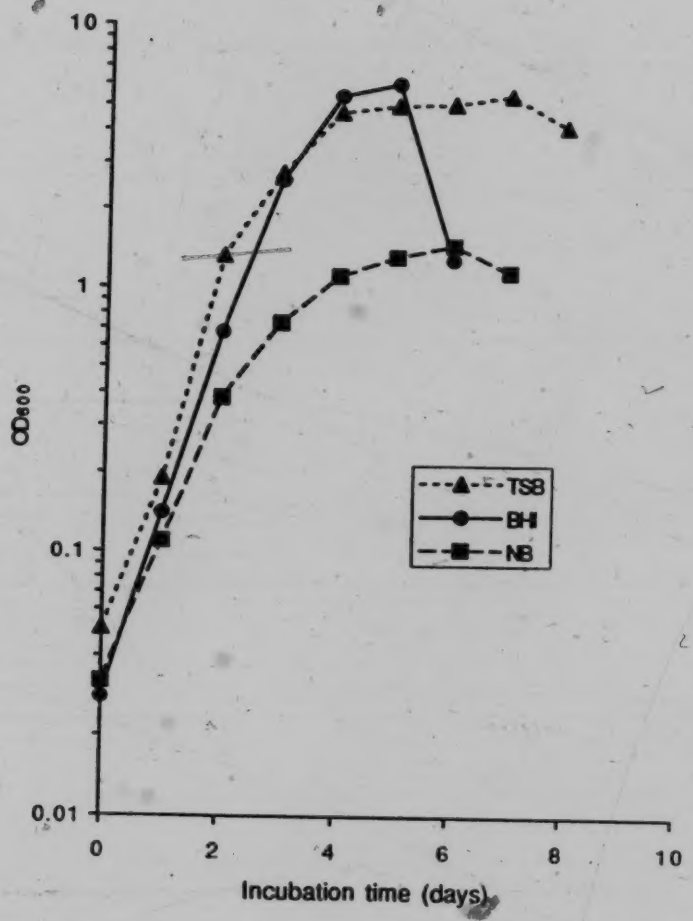


Fig. 11 Growth of *Rhodococcus* sp. strains S1 in trypticase soy broth (TSB), brain heart infusion broth (BHI), and nutrient broth (NB).

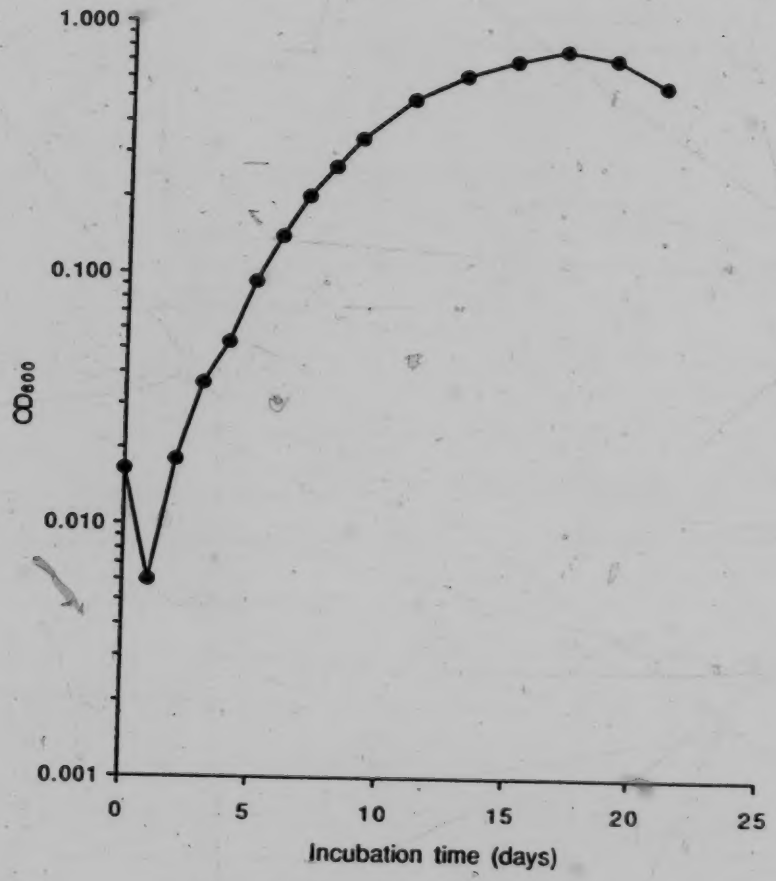


Fig. 12 Growth of *Rhodococcus* sp. strain S1 in anthracene mineral salts medium.

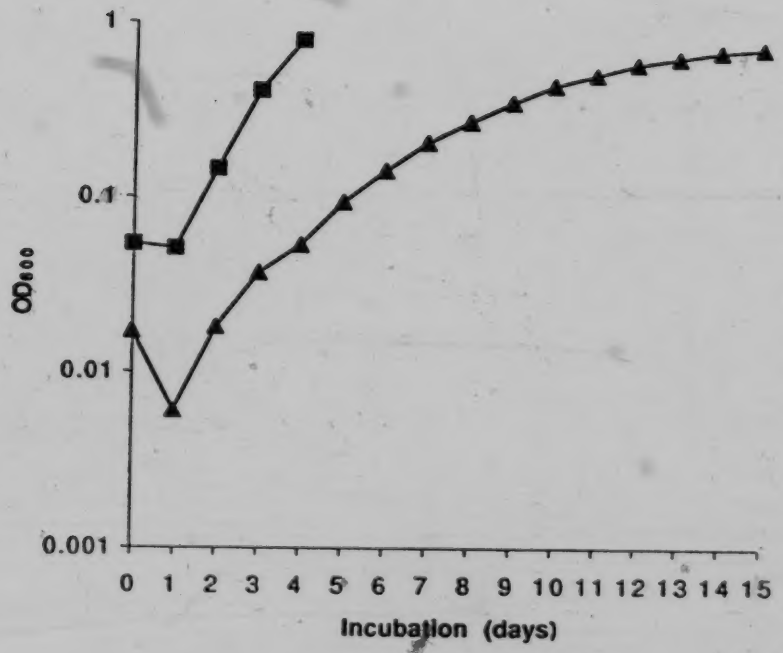
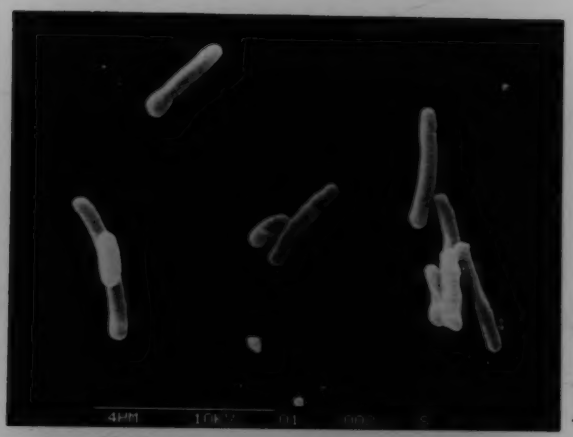
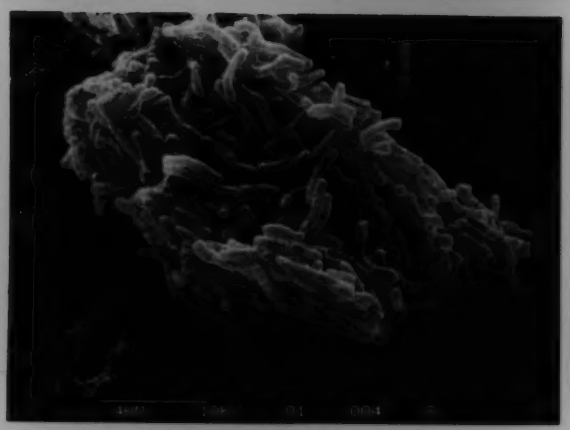


Fig. 13 Growth of *Rhodococcus* S1 on anthracene crystals (▲) or anthracene precipitated from *N,N'*-dimethylformamide (■).

Fig. 14 Scanning electron micrographs of *Rhodococcus* S1 growing in anthracene-MS medium: (A) free cells, (B) clumps in culture supernatant and (C) a thick layer of cells coating the anthracene crystal found in the sediment.



A



B

C

sediment as a thick cell layer (10-50 μm) on the surface of the anthracene crystals (Fig. 14C). This phenomenon implies that the low solubility of anthracene was limiting growth and that the free cells and clumps in the growth medium had been sloughed off from the the crystal surface. However, growth in the presence of a wire coil broke up the clumps, facilitating a maximum sustained growth rate and cell yield.

4.2.2 Growth on various carbon sources.

Rhodococcus S1 was chosen to examine growth of *Rhodococcus* isolates on various individual carbon sources other than anthracene. The strain S1 could utilize several single carbon sources for growth at 1% (for carbohydrates) or 5 mM (for carboxylic acids) final concentration in MS medium, including glucose, mannose, acetate, pyruvate, glutamate, and starch, but not ribose, xylose, sucrose, galactose, lactose, mannitol, sorbitol, citrate, succinate, malate, or lactate.

Growth (OD_{600}) on a variety of PAHs in MS medium was followed over 14 d at 27°C, and growth was limited to anthracene. Under these conditions the following PAHs, at 0.05% final concentration, were unable to serve as carbon source for strain S1: acenaphthene, biphenyl, fluorene, fluoranthene, naphthalene, phenanthrene. Pyrene, chrysene, naphthacene, and 1,2-benzanthracene were tested at 0.02% final concentration and were also unable to serve as carbon source. No growth was observed whether the inocula were noninduced cells, from PCA plates, or induced cells which were initially grown in anthracene-MS medium and filtered through glasswool to remove any residual anthracene.

4.2.3 Mineralization of anthracene, phenanthrene, and naphthalene.

To confirm the PAH substrate specificity and examine potential cometabolism, all three isolates were tested for their ability to mineralize naphthalene, phenanthrene, and

anthracene. Cells grown on anthracene were incubated with ^{14}C-PAH and the rate and extent of $^{14}CO_2$ release was recorded. The results were very similar in that *Rhodococcus* 11S, S1, and 2F1 all mineralized only anthracene but not phenanthrene or naphthalene. The data for *Rhodococcus* S1 are shown in Fig. 15. About 50% of the radioactivity was released as $^{14}CO_2$ during the first 2 h of incubation and reached the maximum (~85%) in 72 h, whereas no $^{14}CO_2$ from radioactive phenanthrene or naphthalene was detected during 4 days of incubation. The control, *Pseudomonas* sp. strain HL4, mineralized 78% of anthracene and 9% of naphthalene, but not phenanthrene during the same incubation period.

Cell counts at inoculation and the end of incubation indicated that no cell number increase had occurred during the 4 days incubation even in the flask where mineralization occurred.

4.2.4 Growth of *Rhodococcus* S1 on substituted anthracenes.

To further investigate the substrate specificity for growth, all commercially available substituted anthracene derivatives were examined for their ability to support growth by themselves or to inhibit growth on anthracene in liquid culture. The inoculum was grown in anthracene-MS medium and single flasks containing 0.05% substituted anthracenes, in the presence or absence of equivalent amounts of anthracene, were used as sole carbon source. Growth was assessed by measuring turbidity at 600 nm. The experiment was repeated with similar results, which are shown in Table 6. Modification of C_2 by methylation, and to a lesser extent chlorination, did not inhibit growth on the substituted anthracene. Modification of the other two positions, C_1 and C_9, by methylation or chlorination, rendered the derivative unusable as a carbon source. Growth on anthracene was inhibited by the presence of 1- and 9-methylanthracene, 2- and 9-chloroanthracene, and 9-bromoanthracene. One explanation of the inhibitory effect of 1- and 9-methylanthracene or 2- and

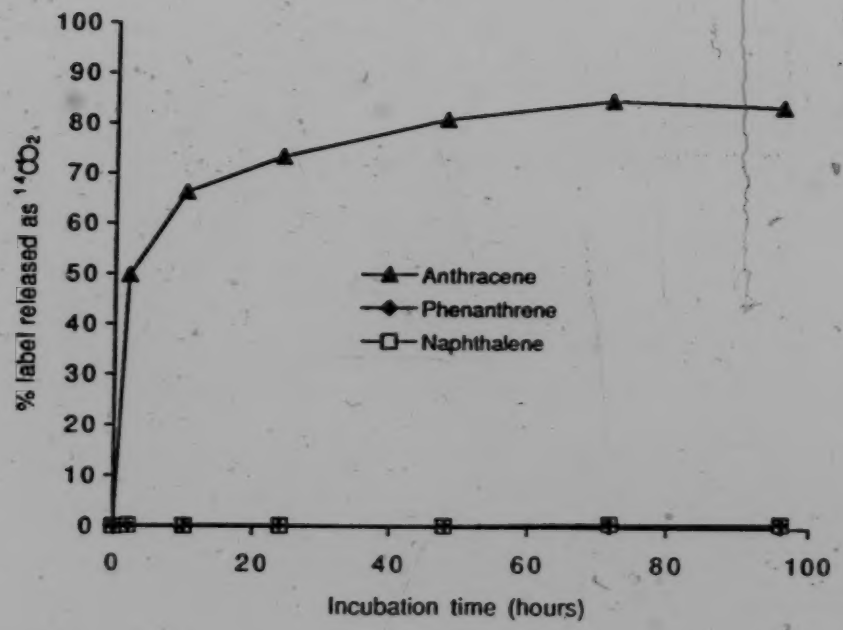


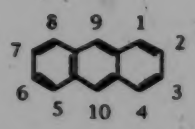
Fig. 15 Mineralization of [^{14}C]anthracene, [^{14}C]phenanthrene, and [^{14}C]naphthalene by *Rhodococcus* sp. strain S1.

Table 6. Effects of substituted anthracenes on growth of *Rhodococcus* sp. strain S1.

Substituted anthracene	Growth on substituted anthracene as sole carbon and energy source	Growth on anthracene in the presence of substituted anthracene
1-Methylanthracene	-	-
2-Methylanthracene	++	++
9-Methylanthracene	-	-
1-Chloroanthracene	-	+/-
2-Chloroanthracene	+/-	+/-
9-Chloroanthracene	-	-
9-Bromoanthracene	-	-

Note: ++, good growth; +, growth; +/-, marginal growth; -, no growth

Chemical structure of anthracene



9-chloroanthracene, or 9-bromoanthracene might be that they block transport of anthracene into the cell. These possibilities were examined by following the uptake of [9-^{14}C]anthracene and 9-methyl-[U-^{14}C]anthracene into washed cells. 9-Methyl-[U-^{14}C]anthracene was taken up by washed cells and it was found that 9-methylanthracene did not inhibit the uptake of [9-^{14}C]anthracene. These data coupled with the narrow PAH growth specificity suggest that it may be the initial, presumably dioxygenase, enzymic attack on anthracene that exhibits a relatively narrow specificity.

4.2.5 Feeding experiments.

The catabolic pathway proposed for anthracene metabolism by *Pseudomonas* spp. (Fig. 3 in section 2.4.1) can be indirectly examined by feeding experiments. If the potential intermediates of the pathway, which are commercially available, can be used as the sole source of carbon and energy for growth of the *Rhodococcus* spp., this would be strong evidence in favor of the *Pseudomonas* pathway existing in *Rhodococcus* as a more general pathway. Since phenanthrene is an isomer of anthracene, intermediate metabolites proposed for the phenanthrene degradative pathway by *Pseudomonas* spp. (see Fig. 5 in section 2.4.2) were also included in the experiments. To examine this, *Rhodococcus* isolates were tested with the potential pathway intermediates of anthracene and phenanthrene proposed for *Pseudomonas* spp.

It was found that at 2.5 mM final concentration in MS medium, 1-hydroxy-2-naphthoic acid, 2-hydroxy-3-naphthoic acid, 2,3-dihydroxynaphthalene, salicylaldehyde, salicylic acid, catechol, *o*-phthalic acid, or protocatechuic acid did not support growth of the *Rhodococcus* isolates pre-grown on anthracene or PCA plates. Therefore, the possibility exists that these test compounds may be unable to enter the cells or alternatively the *Rhodococcus* isolates have a metabolic pathway for anthracene metabolism different from that reported for *Pseudomonas* spp.

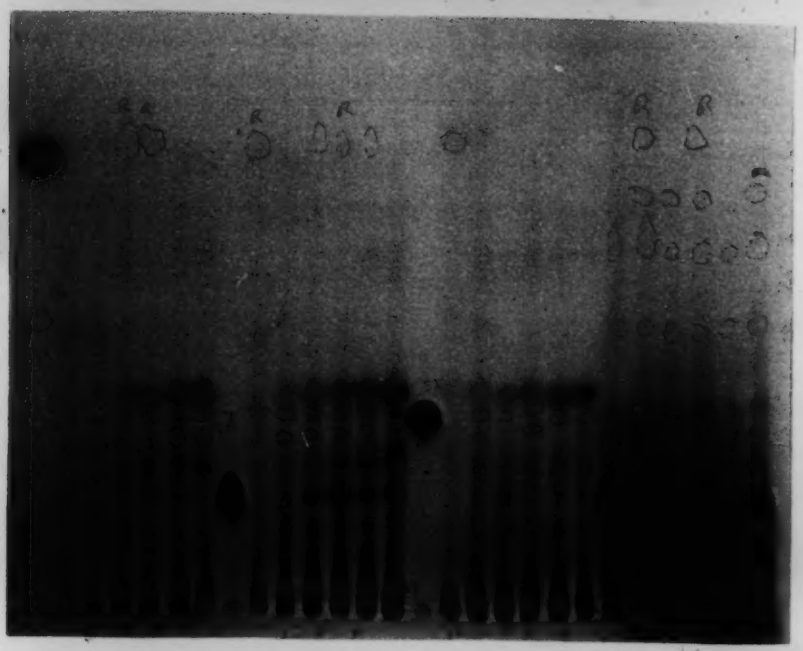
4.2.6 Metabolites from *Rhodococcus* spp. and *Pseudomonas* sp. strain HL4 grown on anthracene-MS medium.

The neutral and acid extracts of growth medium from *Rhodococcus* spp. strain 11S, S1, 2F1, and *Pseudomonas* sp. strain HL4 (control) grown on anthracene-MS medium were analyzed by TLC. Samples were taken from 1-6 weeks and compared with the authentic compounds known to be anthracene metabolites from *Pseudomonas* spp.: 2-hydroxy-3-naphthoic acid and 2,3-dihydroxynaphthalene. Results from TLC revealed that only the acid extract from strain HL4 growth medium contained one metabolic intermediate that resembled 2-hydroxy-3-naphthoic acid, i.e., bright yellow fluorescence under UV (Fig. 16A) and dark greenish blue color after the TLC chromatogram was sprayed with Gibbs' reagent (Fig. 16B). The amount of this compound accumulated up to 4 weeks of incubation before starting to decrease. The acid extract of the isolates 11S, S1, and 2F1 revealed a fluorescent compound in increasing quantities with the increased incubation time to a maximum after 4-6 weeks. However, this compound did not match the authentic 2-hydroxy-3-naphthoic acid or 2,3-dihydroxynaphthalene. TLC studies of the acid extract from 6 weeks growth medium of the isolates 11S, S1, and 2F1 and the acid extract of 4 weeks growth medium of strain HL4 together with the authentic standards 1-hydroxy-2-naphthoic acid, 2-hydroxy-3-naphthoic acid, 2,3-dihydroxynaphthalene, salicyladehyde, salicylic acid, and catechol, is shown in Fig. 17A for UV visualization and after spraying with Gibbs' reagent in Fig. 17B. This confirmed that none of the metabolites present in the growth medium of the *Rhodococcus* isolates 11S, S1, and 2F1 resembled any of the 6 authentic compounds tested. The acid extract of strain HL4 contained only one compound that matched 2-hydroxy-3-naphthoic acid, confirming that *Pseudomonas* sp. strain HL4 uses the previous published pathway to metabolize anthracene but the metabolites from *Rhodococcus* spp. were not consistent with this pathway.

Fig. 16 TLC analysis of the acid extract from weekly samples of *Rhodococcus* spp. strains 11S (lanes 2-7), S1 (lanes 9-14), 2F1 (lanes 16-21), and *Pseudomonas* sp. strain HL4 (lanes 22-27). Growth was for 6 weeks in anthracene-MS medium. Standards were anthracene (lane 1), 2-hydroxy-3-naphthoic acid (lane 8) and 2,3-dihydroxynaphthalene (lane 15): (A) after exposure to short wavelength UV, (B) after spraying with Gibbs' reagent.

A

B

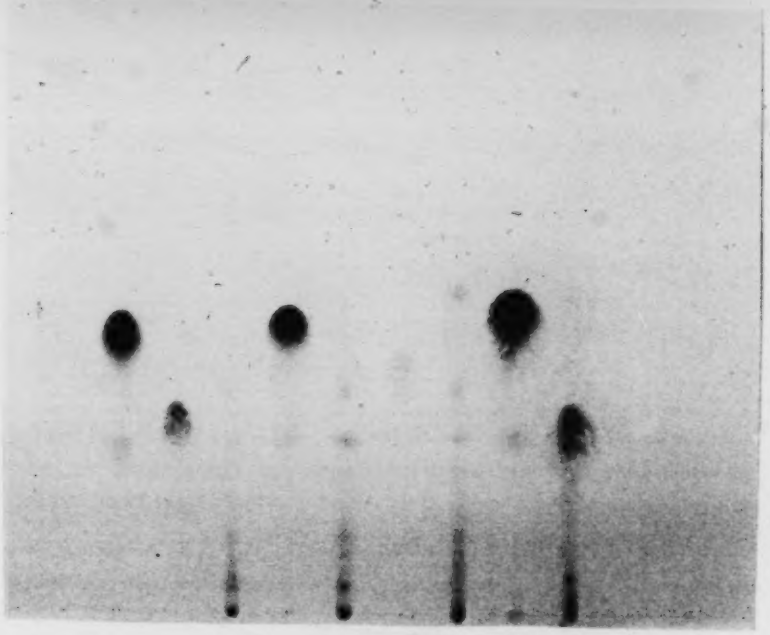


1 • 3 • 5 • 7 • 9 • 11 • 13 • 15 • 17 • 19 • 21 • 23 • 25 • 27

Fig. 17 TLC analysis of the acid extract of culture supernatant of *Rhodococcus* spp. strains 11S (lane 4), S1 (lane 6), and 2F1 (lane 8) after growth in anthracene-MS medium for 6 weeks and culture supernatant of *Pseudomonas* sp. strain HL4 after 4 weeks growth (lane 10). Standard compounds: anthracene (lane 1), 1-hydroxy-2-naphthoic acid (lane 2), 2-hydroxy-3-naphthoic acid (lane 3), 2,3-dihydroxynaphthalene (lane 5), salicylic acid (lane 7), catechol (lane 9), salicyladehyde (lane 11), and the extract of sterile control medium (6 weeks, lane 12): (A) after exposure to short wavelength UV, (B) after spraying with Gibbs' reagent.

A

B



1 2 3 4 5 6 7 8 9 10 11 12

The neutral and acid extracts from *Rhodococcus* spp. and *Pseudomonas* sp. strain HL4 were also analyzed by GC. The retention times of various metabolites were compared with those obtained from the 6 standards. As expected, a compound present in the acid extract of strain HL4 medium after derivatization with BSA, had the same retention time as authentic 2-hydroxy-3-naphthoic acid. Coinjection proved that the retention times were the same. The trimethylsilyl (TMS) derivative of this product produced a mass spectrum (Fig. 18A) that was identical to the spectrum of the TMS derivative of authentic 2-hydroxy-3-naphthoic acid (Fig. 18B).

Anthracene-MS medium extract from *Rhodococcus* S1 did contain several compounds. However, these compounds and their BSA-derivatives did not correspond to any available standards. The neutral and acid extract of *Rhodococcus* S1 were also treated with diazomethane. None of the peaks observed had retention times that matched those of diazomethane derivatives of the standards. Further experimentation was designed to identify the major compound, designated as compound X, found in the neutral extract to determine if it was a novel intermediate in anthracene catabolism by *Rhodococcus* S1.

4.2.7 Neutral extract metabolite of *Rhodococcus* S1 grown on anthracene: compound X.

Rhodococcus S1, when grown on anthracene-MS medium, produced a neutral metabolite, compound X, as a major metabolite which did not match any of the commercially available standards. This compound showed a mass spectrum with a molecular ion (M^{+}) at *m/z* 212 and the next fragment ion at *m/z* 194 (M^{+} -H_2O) (Fig. 19 A). High resolution GC-MS of compound X gave an observed mass of 212.08 consistent with a chemical formula of $C_{14}H_{12}O_2$ which in turn might correspond to anthracene dihydrodiol based on the following evidence. The mass spectrum of the TMS derivative of compound X had a M^{+} at *m/z* 356 (Fig. 19 B) indicating that two molecules of BSA

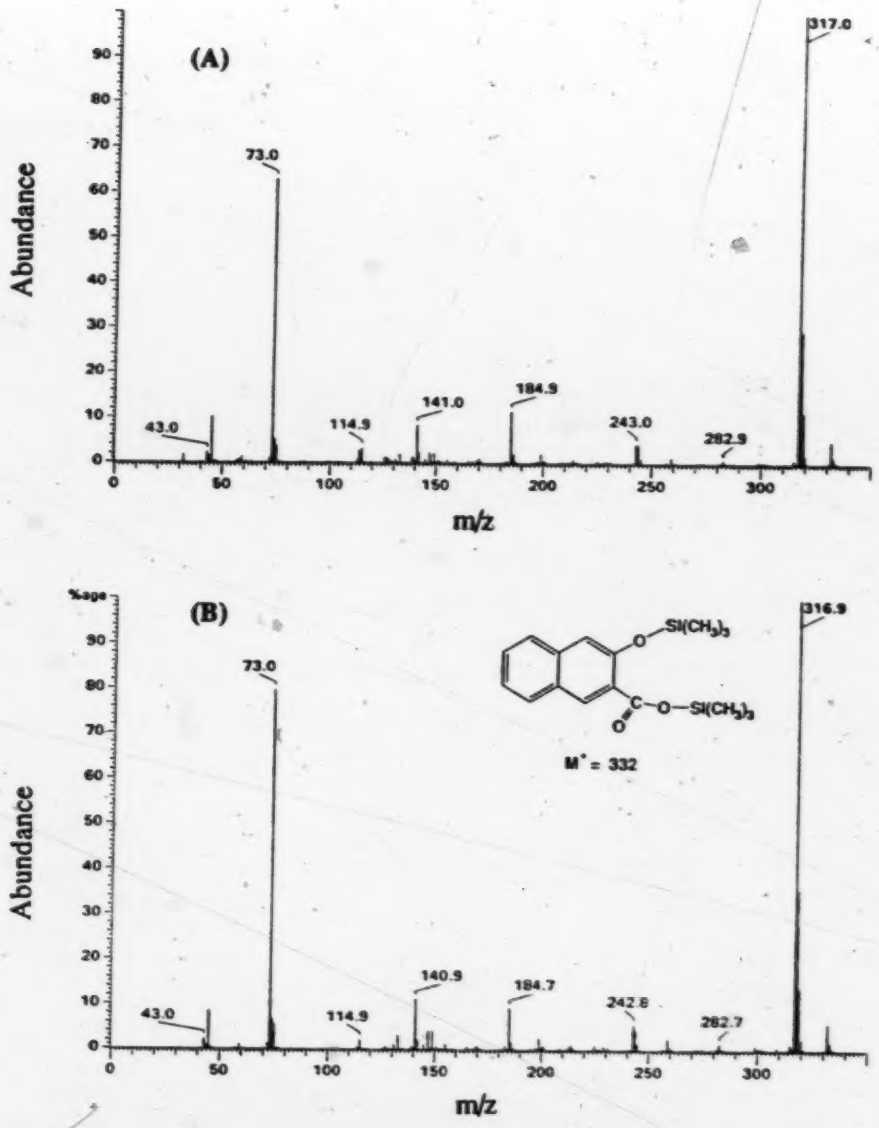


Fig. 18 Mass spectra of the metabolite formed from the degradation of anthracene by *Pseudomonas* sp. strain HL*A*. (A) TMS derivative of an acid-extracted metabolite. (B) TMS derivative of the authentic 2-hydroxy-3-naphthoic acid.

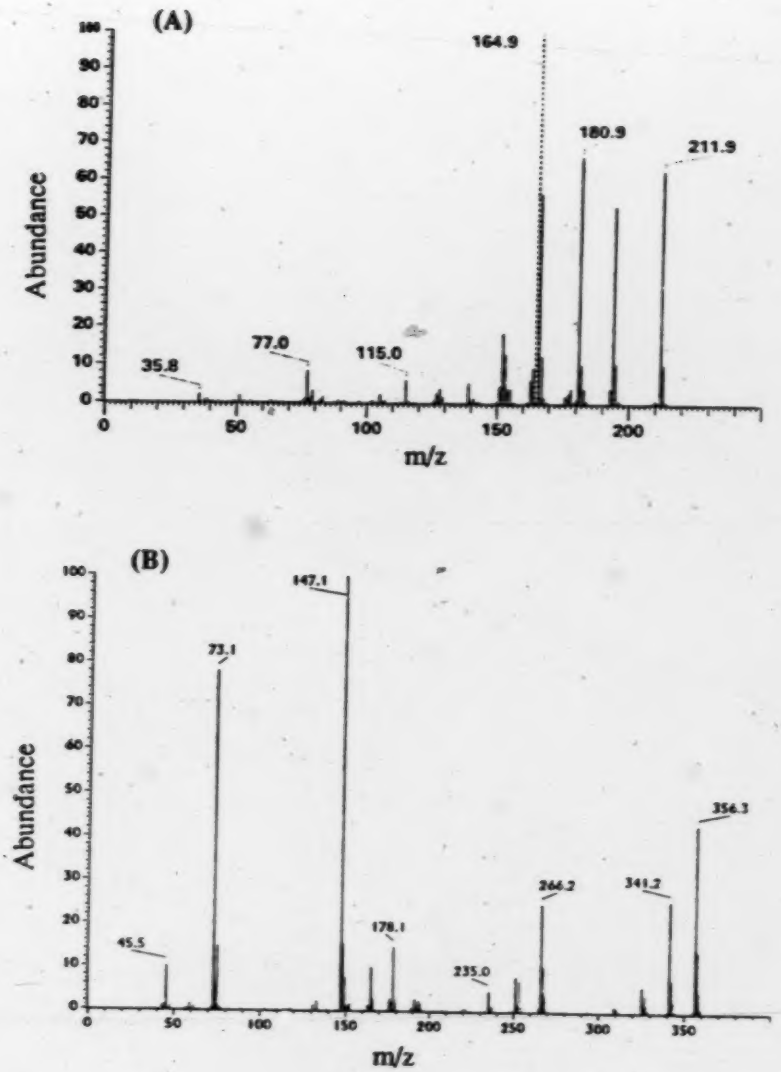


Fig. 19 Mass spectra of compound X (A) and its TMS derivative (B) found in the neutral extract of *Rhodococcus* S1.

reacted with two hydroxyl groups on the compound. High resolution GC-MS of the TMS derivative of compound X gave an observed mass of 356.15321 which was consistent with a chemical formula of $C_{20}H_{28}O_2Si_2$.

4.2.8 Anthracene 1,2-dihydrodiol from *Pseudomonas fluorescens* LP6a (pLP6a :: *Tn*5) mutant D1.

To confirm compound X as anthracene 1,2-dihydrodiol, the presumed product of anthracene dioxygenase, anthracene 1,2-dihydrodiol was prepared from *P. fluorescens* LP6a mutant D1. The bacterium induced by salicylic acid prior to shaking with anthracene yielded a neutral extract dihydrodiol. Mass spectrum of the neutral extract revealed a compound with a M^+ at *m/z* 212 which corresponded to anthracene dihydrodiol (Fig. 20A). The next fragment ion at *m/z* 194 (M^+ -H_2O) was the base peak. The BSA-treated neutral extract showed a TMS derivative of the compound with a M^+ at *m/z* 356, indicating that two molecules of TMS reacted with two hydroxyl group of anthracene dihydrodiol (Fig. 20B).

The data described in section 4.2.7 indicated that compound X was also a dihydrodiol. However, coinjection of the neutral extract of *P. fluorescens* LP6a mutant D1 before and after BSA treatment with the neutral extract of *Rhodococcus* S1 before and after BSA treatment clearly showed that compound X was not anthracene 1,2-dihydrodiol because they had different retention times.

4.2.9 Chemical dehydration of compound X from *Rhodococcus* S1 to compound Y.

Compound X in the neutral extract of *Rhodococcus* S1 was depleted after chemical dehydration, yielding another peak designated as compound Y. This compound had a mass spectrum with a M^+ at *m/z* 194 (base peak) and the next abundant ion at *m/z* 165 (M^+

-CHO) which is usually found in aromatic alcohols (Silverstein *et al.* 1991) (Fig. 21A). Chemical ionization mass spectrometry confirmed that 194 was the molecular ion. The molecular weight of compound Y obtained from high resolution GC-MS analysis was consistent with the chemical formula $C_{14}H_{10}O$. GC-FTIR analysis of compound Y showed an absorption of hydroxyl group at 3648 cm^{-1} (Fig. 22).

After derivatization with BSA, compound Y yielded only one peak of its TMS ether. Its mass spectrum had a M^+ at *m/z* 266 (Fig. 21B). High resolution of the TMS ether of compound Y gave a molecular weight consistent with a chemical formula: $C_{17}H_{18}OSi$.

4.2.10 Chemical dehydration of anthracene dihydrodiol from *P. fluorescens* LP6a mutant D1.

The neutral extract of *P. fluorescens* LP6a mutant D1, after chemically dehydration, yielded a GC peak designated compound A (Fig. 23A). Mass spectrum of compound A had a M^+ at *m/z* 194 (base peak) and the next abundant ion at *m/z* 165 (M^+ -CHO) indicating the loss of 29 mass unit that normally occurred in aromatic alcohols (Fig. 23B). GC-FTIR analysis of this compound showed an absorption at 3649 cm^{-1} consistent with a hydroxyl group (Silverstein *et al.* 1991) (Fig. 24).

After derivatization with BSA, compound A yielded two peaks of TMS ethers with 1.5 min difference in retention time, named compound A1 and A2 (Fig. 25). They both had a similar mass spectrum showing the M^+ at *m/z* 266 as the base peak (Fig. 25). Molecular weights of compounds A1 and A2 obtained from high resolution GC-MS analysis were consistent with the chemical formula $C_{17}H_{18}OSi$. Prior to derivatization, A1 and A2 had coeluted during GC analysis and had been mistaken as a single compound A. Thus, dehydration of *P. fluorescens* LP6a mutant D1 anthracene dihydrodiol yielded two isomers of anthrol.

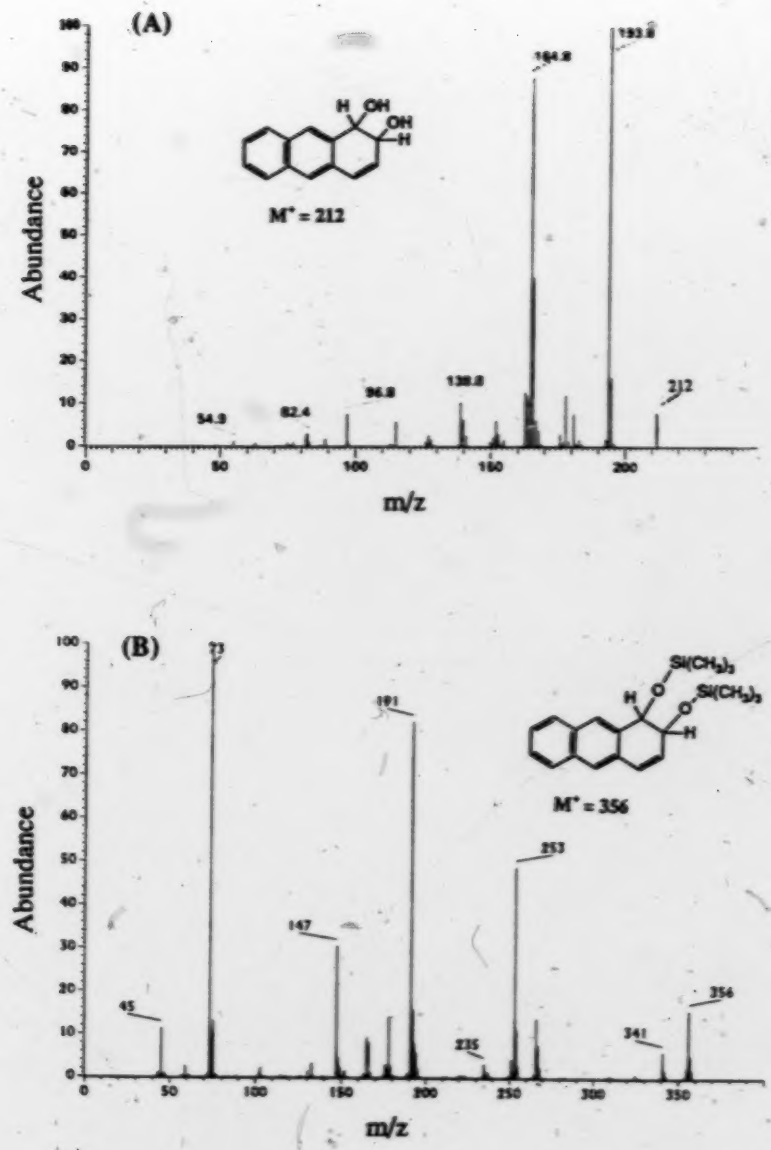


Fig. 20 Mass spectra of anthracene 1,2-dihydrodiol (A) and its TMS derivative (B) found in the neutral extract of *P. fluorescens* LP6a mutant D1.

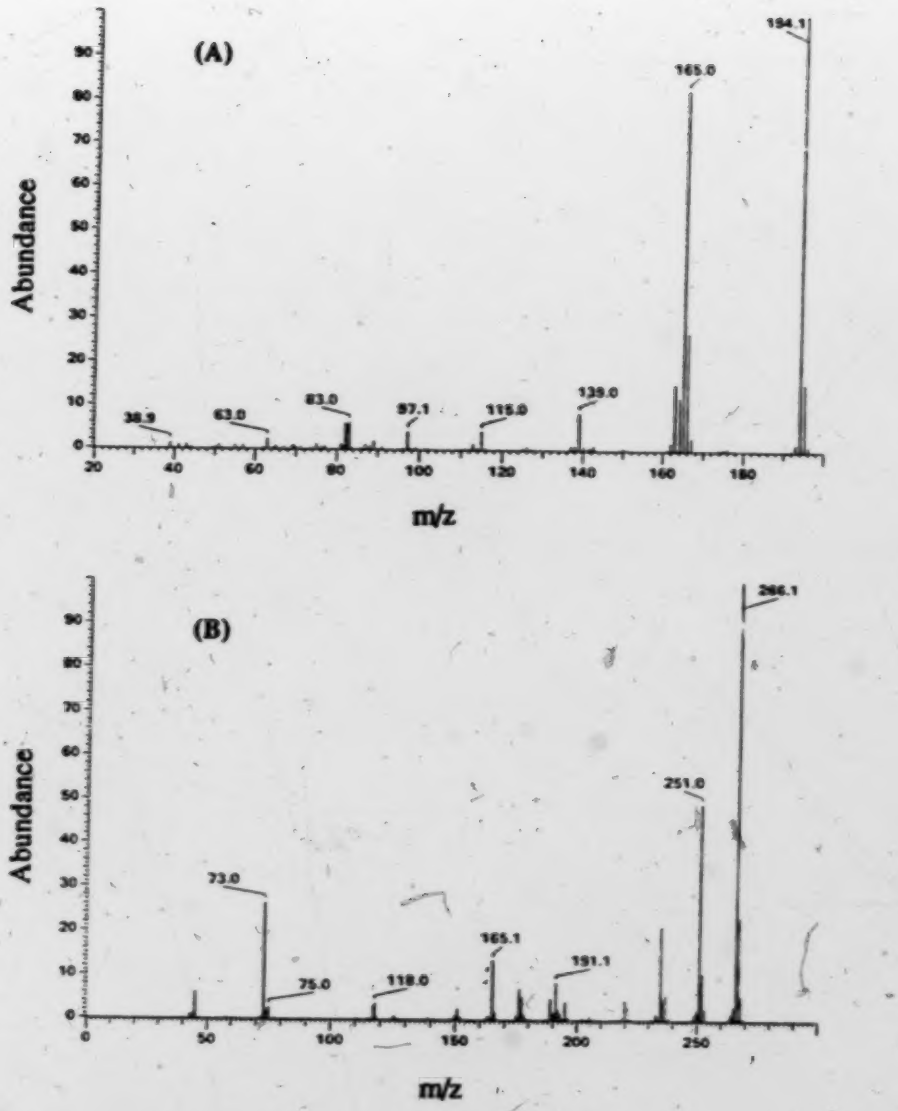


Fig. 21 Mass spectra of compound Y, a chemically dehydrated product of compound X (A) and its TMS derivative (B).

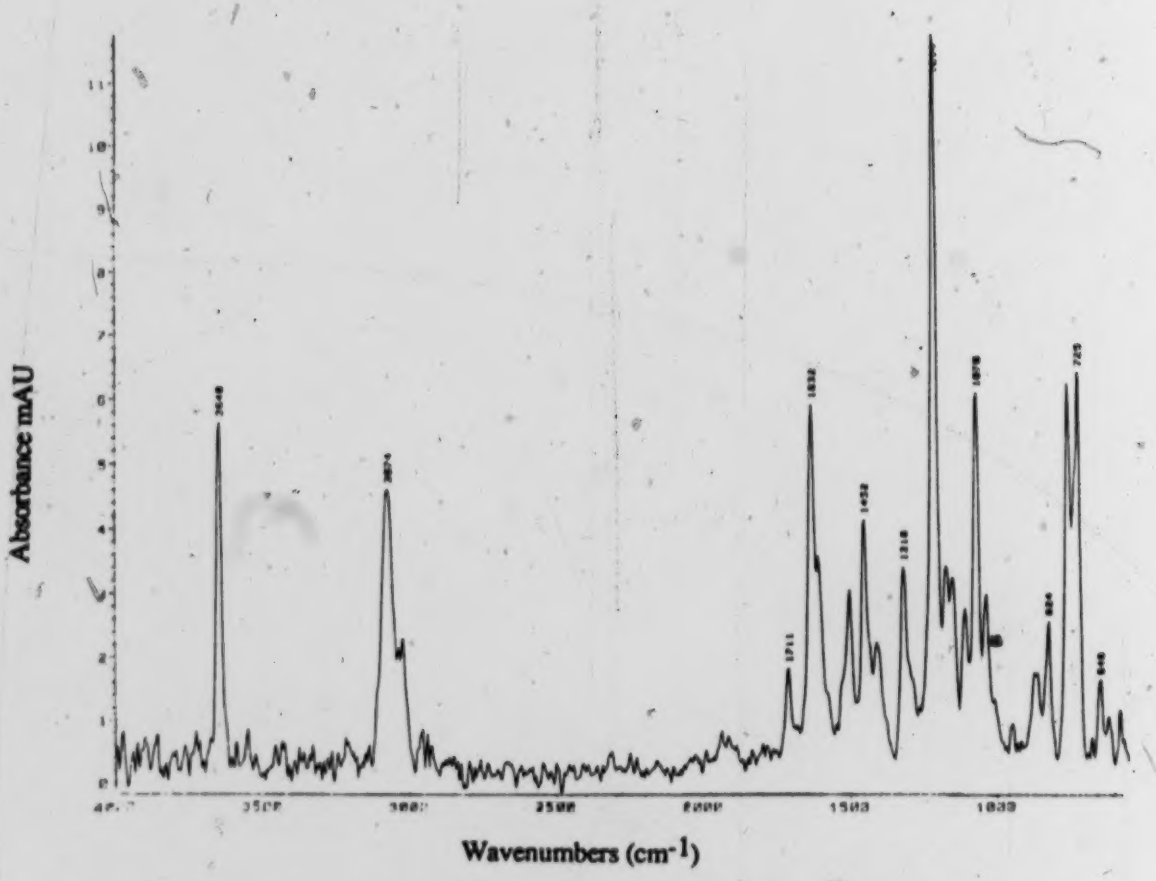


Fig. 22 GC-FTIR spectrum of compound Y, a chemically dehydrated product of compound X.

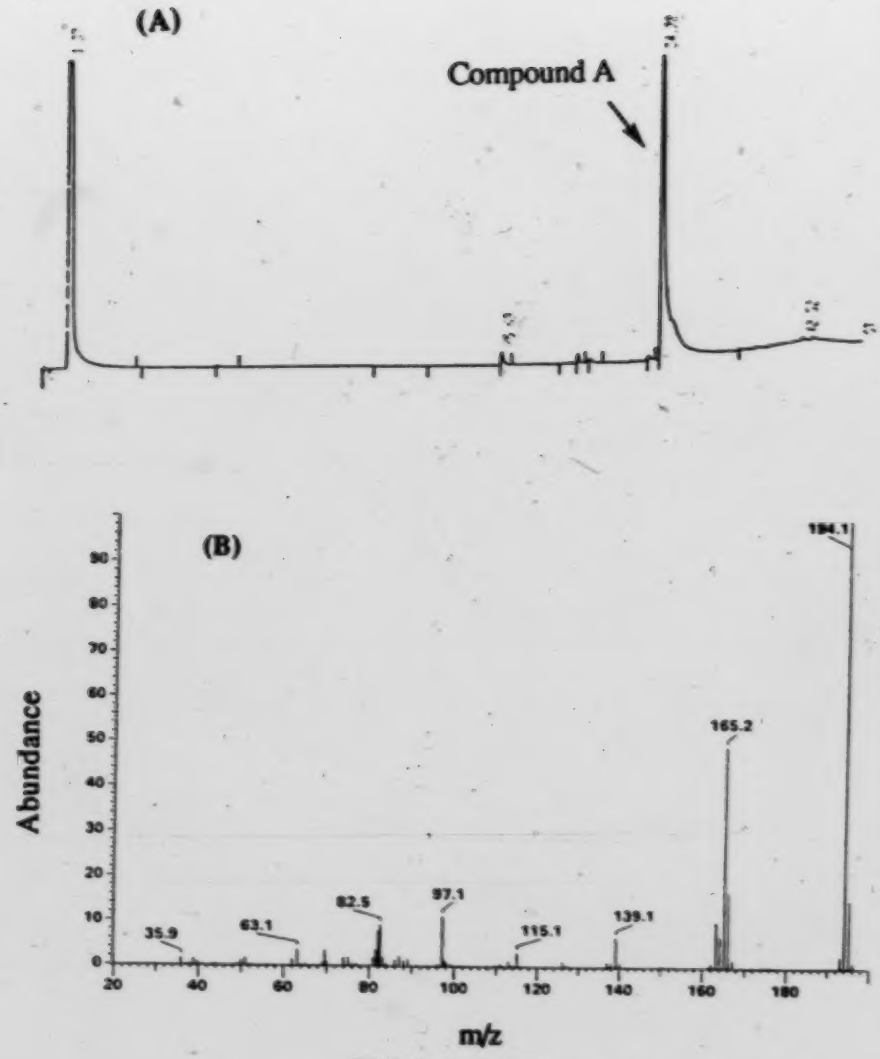


Fig. 23 GC chromatogram of the chemically dehydrated neutral extract of *P. fluorescens* LP6a mutant D1 showing the compound A peak (A) and its mass spectrum (B).

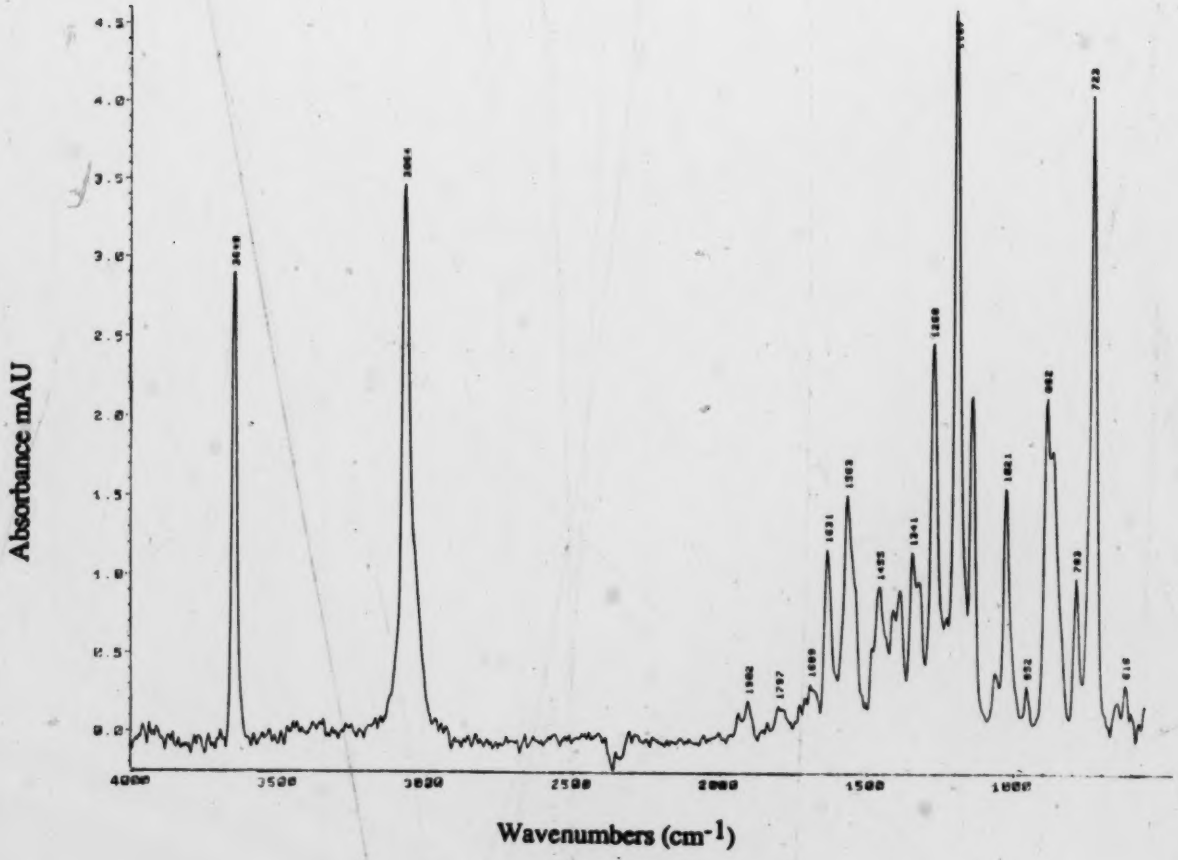


Fig. 24 GC-FTIR spectrum of compound A, a chemically dehydrated product of the neutral extract of *P. fluorescens* LP6a mutant D1.

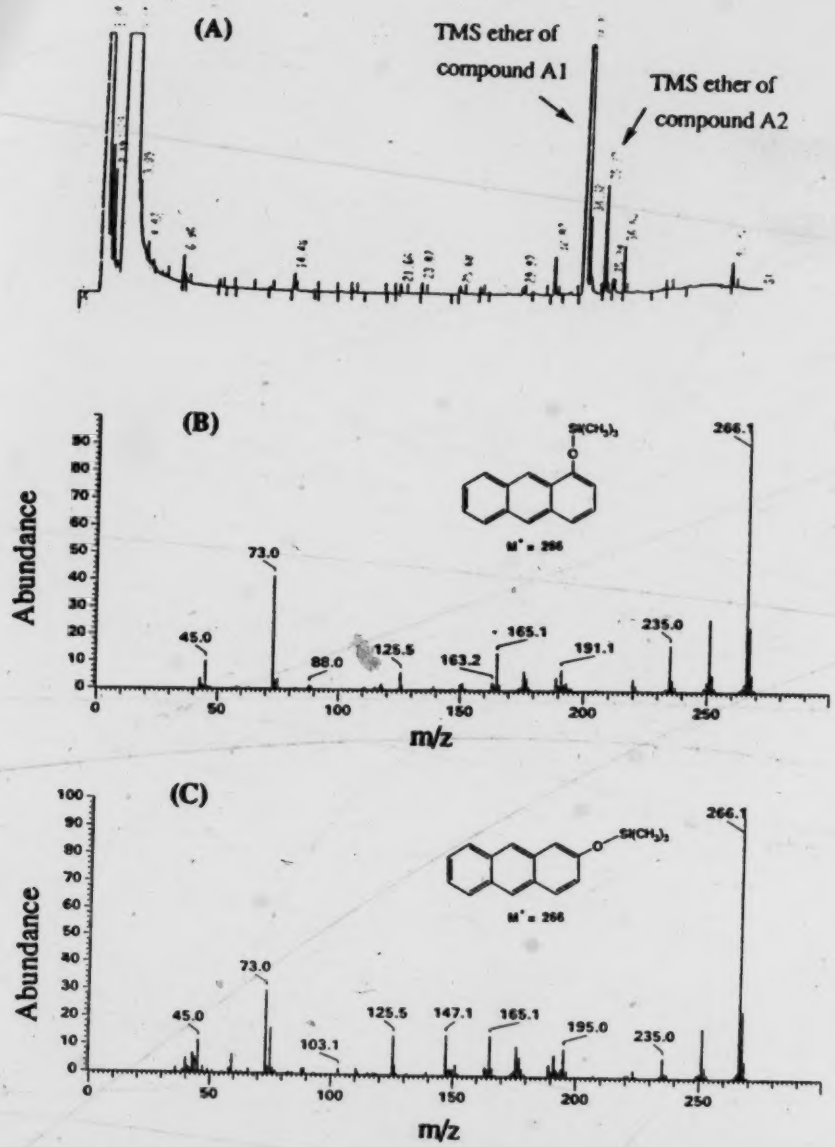


Fig. 25 GC chromatogram of the chemically dehydrated neutral extract of *P. fluorescens* LP6a mutant D1 after treating with BSA showing TMS ether of compound A1 and A2 (A), the mass spectra of TMS ether of compound A1 (B) and A2 (C).

4.2.11 Comparison of the dehydrated products of the neutral extracts from *P. fluorescens* LP6a mutant D1 and *Rhodococcus* S1.

Coinjection of the dehydrated products of the neutral extracts from *P. fluorescens* LP6a mutant D1 and *Rhodococcus* S1 was performed using GC-FID and GC-MS methods. Even though compound Y, a dehydrated product of neutral extract of *Rhodococcus* S1, had a mass spectrum similar to that of compound A, a dehydrated product of neutral extract of strain LP6a-D1, they did not coelute. Likewise, when BSA-treated dehydrated products A1 and A2 of the neutral extract from strain LP6a-D1 were coinjected with that from *Rhodococcus* S1 (compound Y), the TMS ether of compound Y did not coelute with derivatized compounds A1 or A2. Thus, since the product Y from dehydration of metabolite X from *Rhodococcus* S1 did not have the same retention time as the dehydration products A1 and A2 from anthracene 1,2-dihydrodiol produced in *Pseudomonas* culture, metabolite X is not anthracene 1,2-dihydrodiol.

4.2.12 Chemically synthesized 1-, 2-, and 9-anthrol.

To confirm the above conclusion, the individual anthrol isomers were chemically synthesized for comparison. The 1- and 2-anthrols obtained from chemical syntheses had GC retention times of 32.11 and 33.25 min, respectively. GC-MS analyses of 1-anthrol (Fig. 26) and 2-anthrol (Fig. 27) showed the same fragmentation pattern. TMS ether of 1-anthrol, Tr = 31.27 min (Fig. 28) and 2-anthrol, Tr = 33.42 min (Fig. 29) also had similar GC-MS fragmentation patterns. GC-FTIR analysis of 1-anthrol (Fig. 30) and 2-anthrol (Fig. 31) showed an absorption of hydroxyl group at 3649 cm^{-1} and 3647 cm^{-1} respectively. Both standards had slightly different FTIR spectra.

The 9-anthrol which was produced from 9-anthrone was normally unstable. GC-FTIR analysis of the synthesized compound showed strong absorption at 1685 cm^{-1}, indicating the presence of carbonyl instead of hydroxyl group (Fig. 32). This result

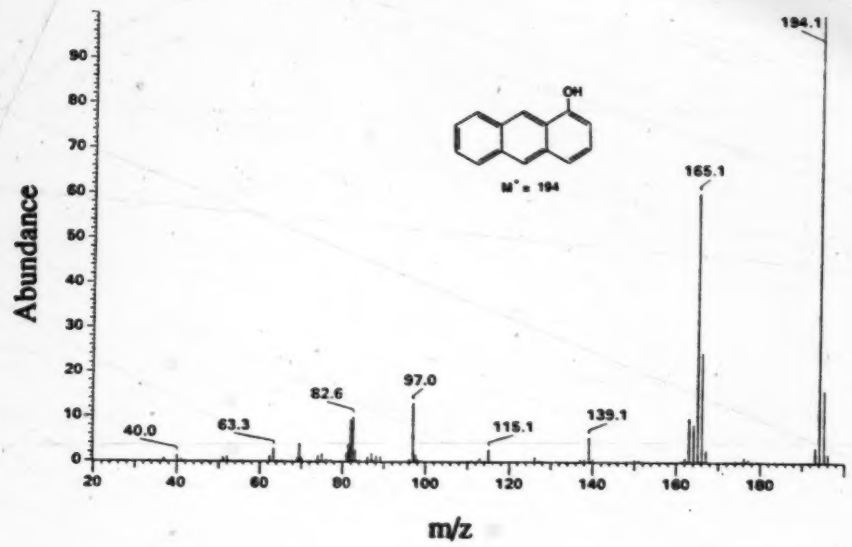


Fig. 26 The mass spectrum of 1-anthrol.

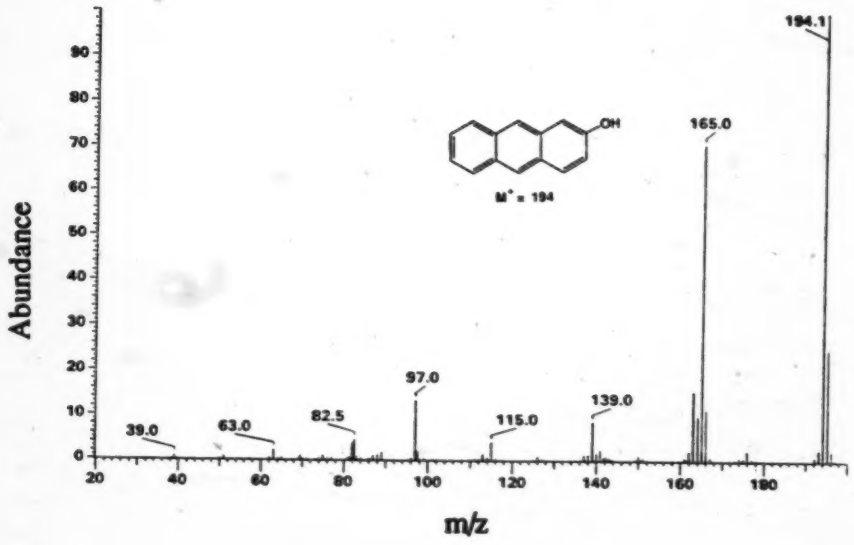


Fig. 27 The mass spectrum of 2-anthrol.

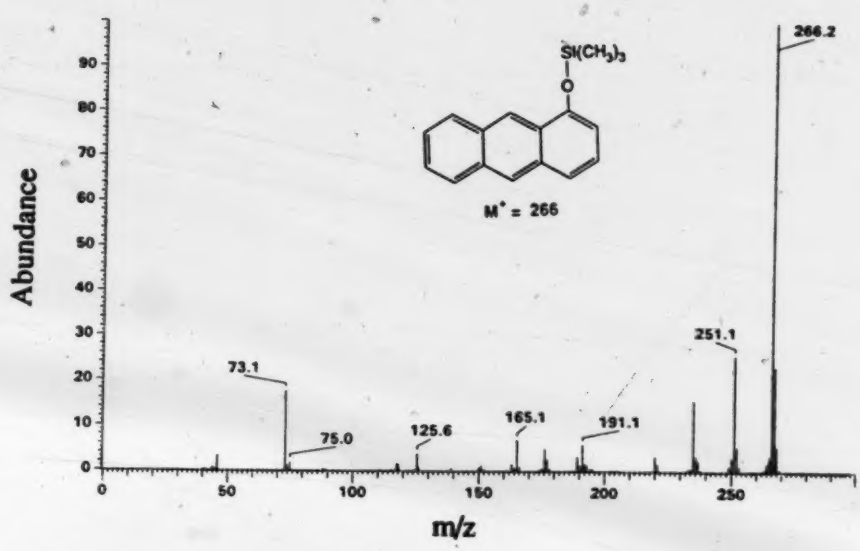


Fig. 28 The mass spectrum of TMS ether of 1-anthrol.

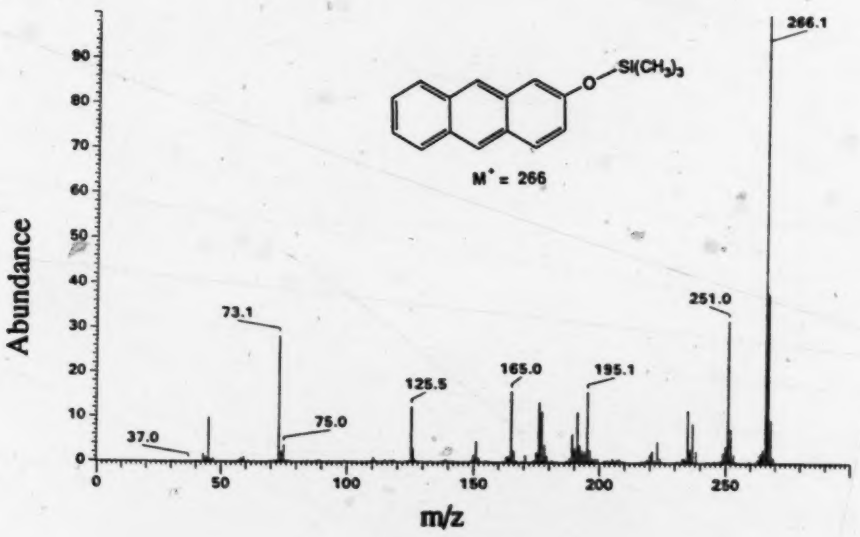


Fig. 29 The mass spectrum of TMS ether of 2-anthrol.

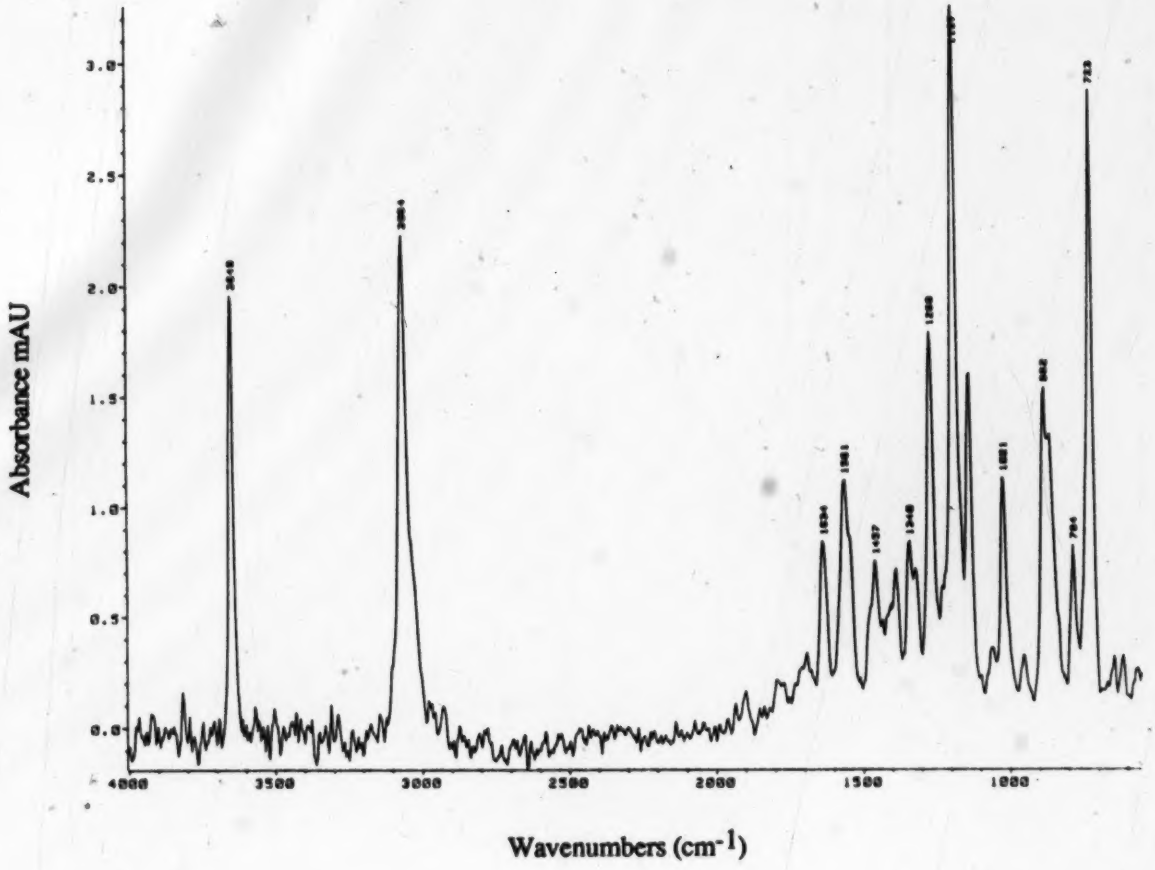


Fig. 30 FTIR spectrum of 1-anthrol.

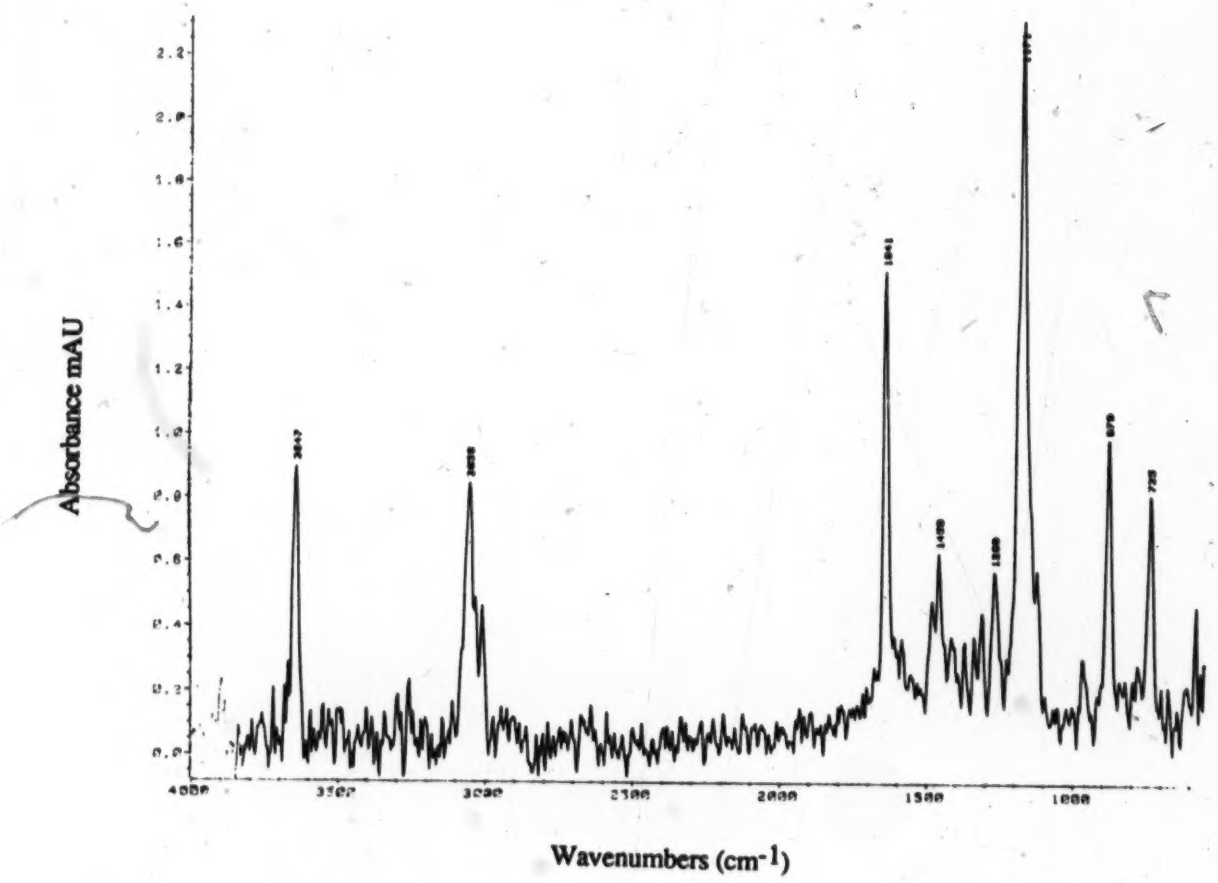


Fig. 31 FTIR spectrum of 2-anthrol.

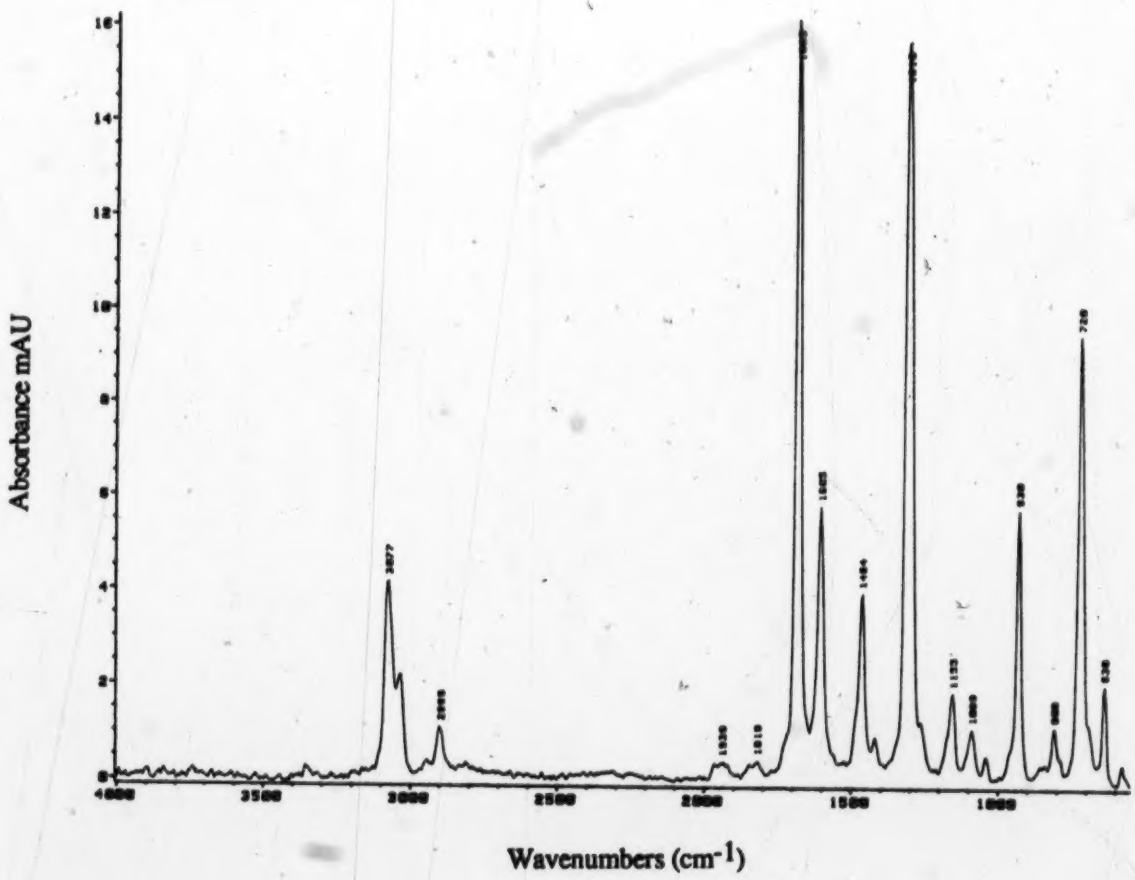


Fig. 32 FTIR spectrum of 9-anthrone.

suggested that the chemically synthesized 9-anthrol preferentially tautomerized to 9-anthrone unless it was derivatized with some reagent such as BSA. Therefore, the TMS ether of 9-anthranol was used in these experiments. The TMS derivative of 9-anthrol had similar GC-MS spectrum (Fig. 33) as those of the TMS ethers of 1- and 2-anthrol (Fig. 28, 29).

4.2.13 Identification of unknown compound A1, A2, and Y.

The unknown compounds were identified by comparing their GC retention times, mass and FTIR spectra with the standard 1-, 2-, and 9-anthrol. From coinjection, the TMS ether of compound A1 coeluted with the TMS ether of standard 1-anthrol. As expected, the TMS ether of compound A2 also coeluted with the TMS ether of standard 2-anthrol. These results confirmed that compound A was a mixture of compounds A1 and A2 which were 1- and 2-anthrol respectively. Since their retention time was very close (~ 14 seconds apart); without derivatization they appeared together as a single peak with a small shoulder. After derivatization with BSA they eluted as single peaks at retention times greater than 2 min apart. Comparing the peak areas of those 2 peaks indicated that chemical dehydration of anthracene dihydrodiol from *P. fluorescens* LP6a mutant D1 yielded 1-anthrol (91 %) in preference to 2-anthrol (9 %). The dehydrated products 1- and 2-anthrol obtained from anthracene dihydrodiol confirmed that strain LP6a-D1 produced anthracene 1,2-dihydrodiol as the initial metabolite as reported in the proposed pathway.

Compound Y was coinjected into the GC with the standard 1- and 2-anthrol. Coinjection of the TMS ether of compound Y with the TMS ethers of 1-, 2-, and 9-anthrol was also performed. It was shown that compound Y and its TMS ether did not coelute with the standards. Thus, compound Y was not an anthrol and could not have been formed from chemical dehydration of an anthracene dihydrodiol as compound X was presumed to be (section 4.2.7).

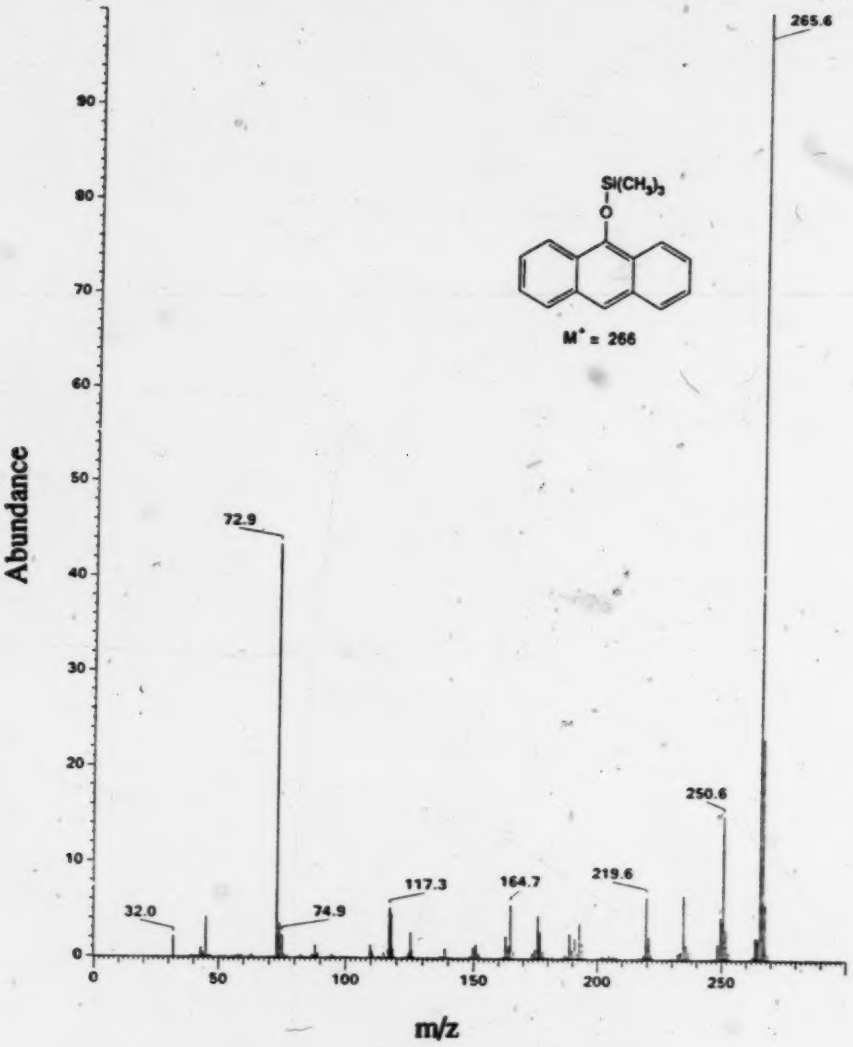
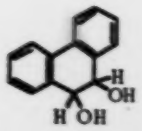


Fig. 33 The mass spectrum of TMS ether of 9-anthrol.

As a further step towards the identification of compound Y, an attempt to purify the compound by alkali extraction of spent growth medium followed by HPLC, for NMR analysis was performed. Several hundred litres of cells and medium were grown over a period of 1.5 years but poor recovery from purification stages and metabolite instability caused this line of investigation to be unproductive. The identification of compound Y was also attempted using other commercially available standards related to anthrol. Since compound X, the neutral metabolite from *Rhodococcus* S1 grown on anthracene could not be anthracene dihydrodiol even though the molecular weight and chemical formula were consistent with a dihydrodiol of a three-ring PAH, the most likely possibility seemed to be that this metabolite came from phenanthrene. This PAH was present as a <1 % contaminant in commercially available anthracene, labelled as ">99%", used in the growth medium. Since 9-phenanthrol is the only phenanthrol isomer that is commercially available, it was used for comparison with the unknown compound Y.

Coinjection of compound Y and authentic 9-phenanthrol revealed that they coeluted. The mass spectra of compound Y and an authentic 9-phenanthrol are shown in Fig. 34A & 34B, respectively. Likewise, the TMS ethers of compound Y (Fig. 35A) and 9-phenanthrol (Fig. 35B) also coeluted and they showed the same mass spectrum. These results confirmed that the metabolite X in the neutral extract of *Rhodococcus* S1, was dehydrated to give 9-phenanthrol, suggesting that the bacterial oxygenase enzyme oxidized phenanthrene at the 9 and 10 position to give phenanthrene 9,10-dihydrodiol as shown below.



Phenanthrene 9,10-dihydrodiol

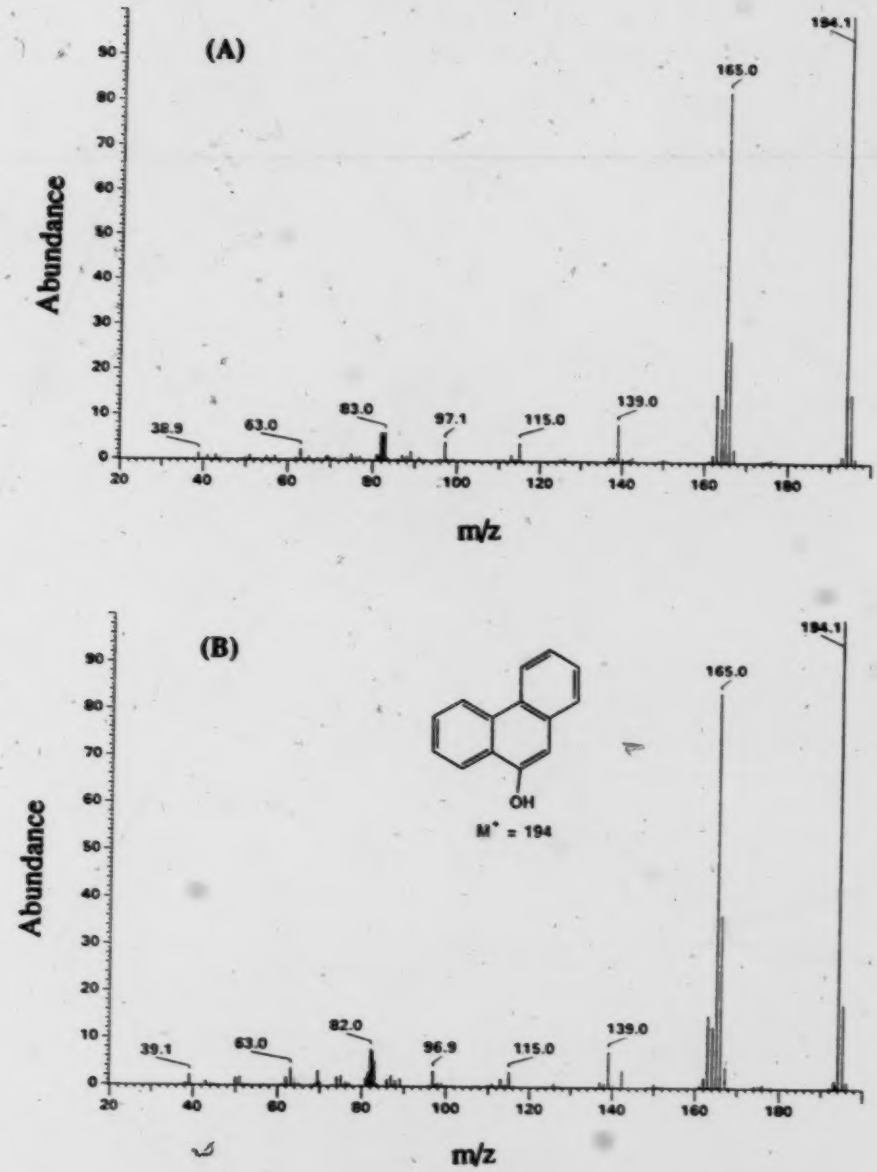


Fig. 34 Mass spectra of a chemically dehydrated product of the neutral extract of *Rhodococcus* S1, compound Y (A) and the standard 9-phenanthrol (B).

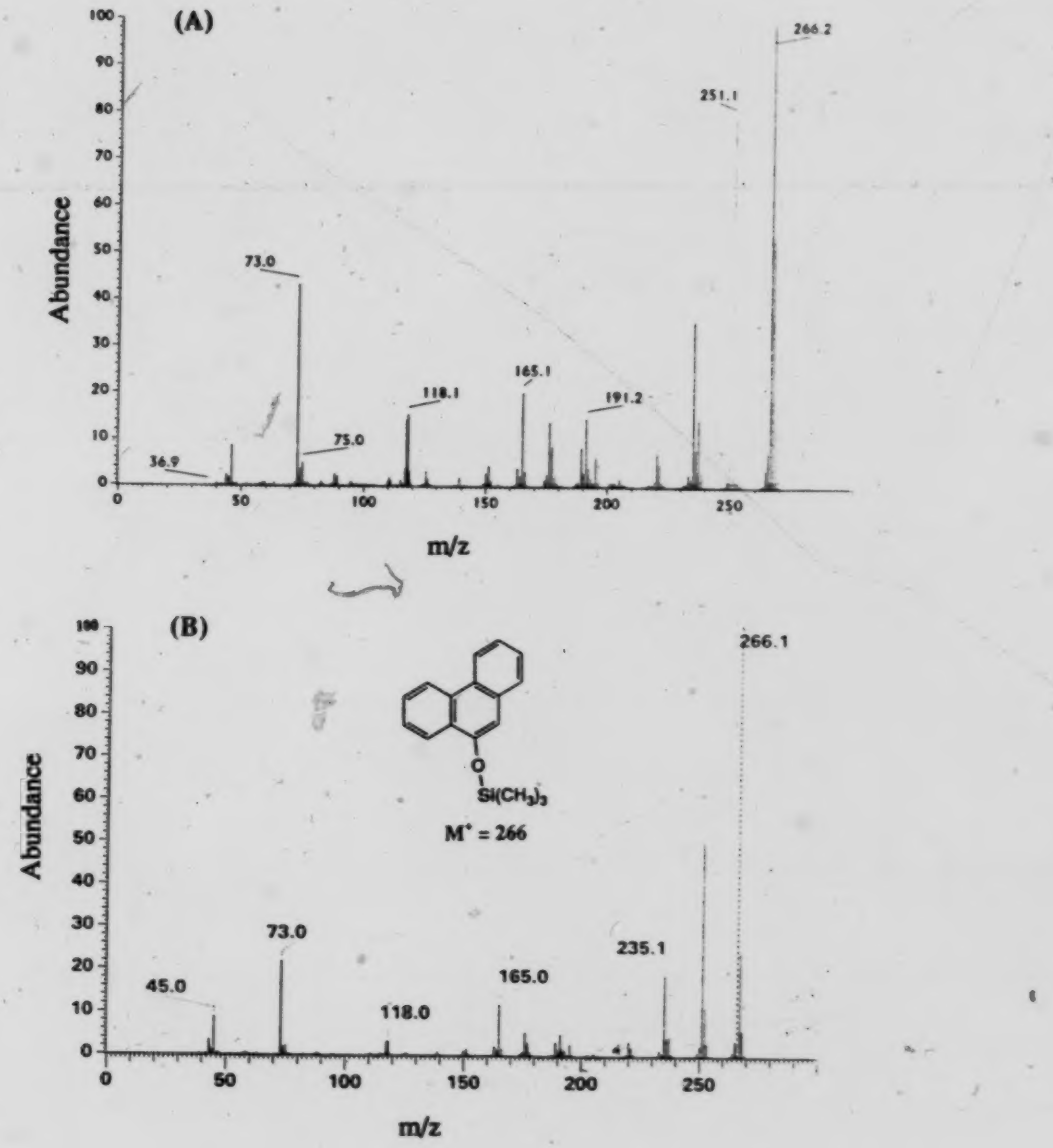


Fig. 35 Mass spectra of the TMS ether of compound Y (A) and a standard 9-phenanthrol (B).

Compound X was also derivatized with another derivatizing reagent, butaneboronic acid (BTB). GC-MS analysis of BTB derivative of compound X showed a mass spectrum with a M^+ at *m/z* 278 which showed fragment ions corresponding to stepwise degradation of the butyl side-chain (Fig. 36). The BTB derivatized compound X with MW 278 indicated that one molecule of BTB reacted with two hydroxyl groups on the phenanthrene dihydrodiol.

Thus, compound X is phenanthrene 9,10-dihydrodiol formed by bacterial oxidation of the small (<1%) phenanthrene present in analytical grade anthracene used in the growth medium.

4.2.14 Confirmation that compound X was derived from phenanthrene.

Analysis of the >99% pure anthracene used in this study by GC confirmed that the <1% impurity was phenanthrene. Increased production of compound X was demonstrated by increasing the phenanthrene content of the growth medium from 1% to 5% of the anthracene level. Analysis of the neutral extract metabolites by GC-FID using dibenzothiophene as an internal standard showed that with the addition of phenanthrene, compound X was produced in about 33 times greater amounts than without addition (Fig. 37). This confirmed that compound X was a metabolite from phenanthrene.

4.2.15 Production of phenanthrene 1,2- and 3,4-dihydrodiols.

To confirm that compound X from *Rhodococcus* S1 was phenanthrene 9,10-dihydrodiol, and that it would not by chance coelute with other phenanthrene dihydrodiol isomers, its characteristics were compared with phenanthrene 1,2- and 3,4-dihydrodiols produced by *P. fluorescens* LP6a mutant D1 (Foght and Westlake 1996). The neutral extract of this bacterium revealed two nearly coeluting peaks (designated I & II) in the GC

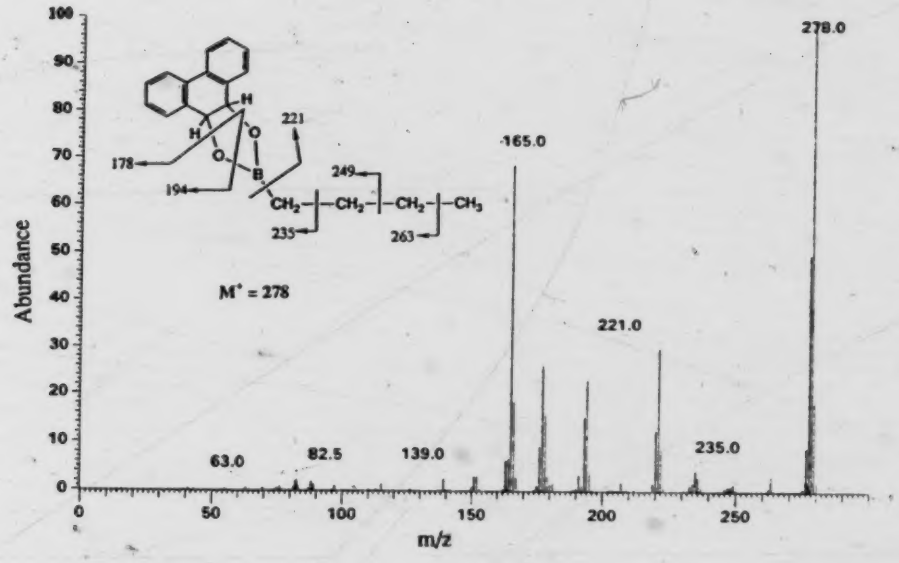


Fig. 36 Mass spectrum of the BTB derivative of compound X in the neutral extract of *Rhodococcus* S1.

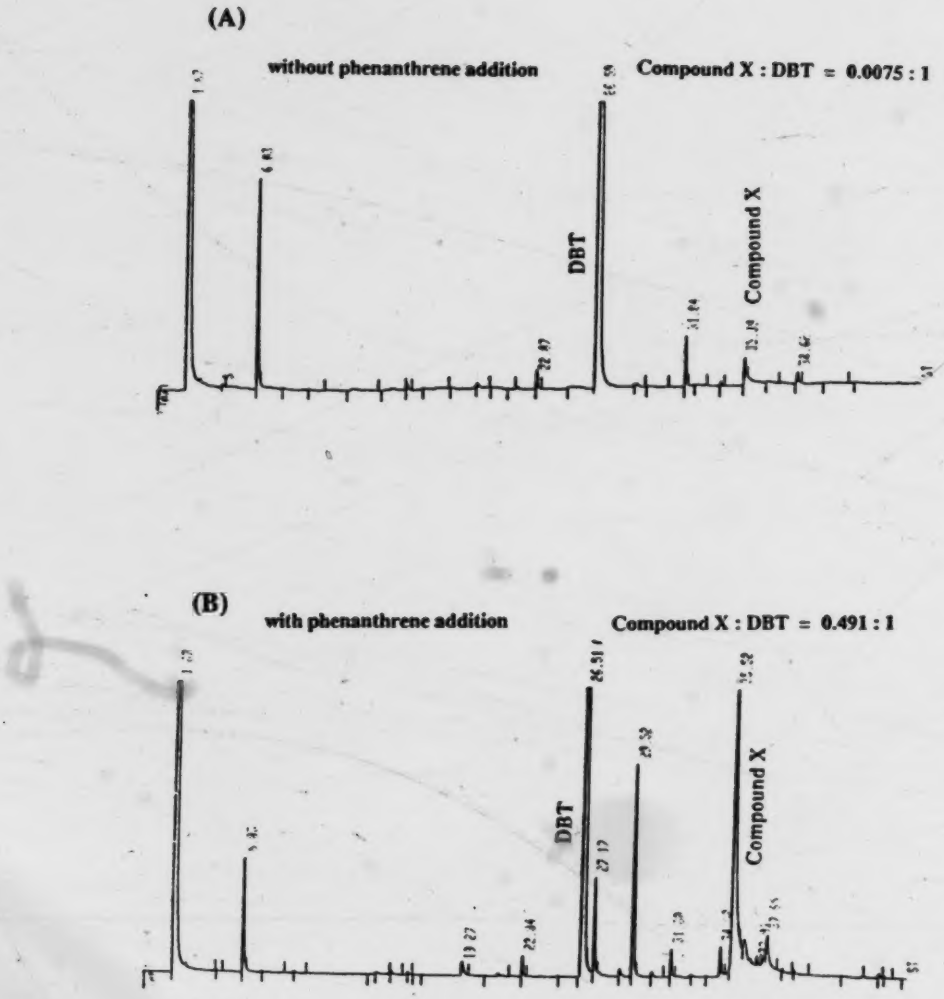


Fig. 37 GC chromatograms of the neutral metabolites from *Rhodococcus* S1 when grown on the 99% pure anthracene which contained 1% phenanthrene as the impurity (A) and when phenanthrene was increased to 5% of anthracene (B)

chromatogram (Fig. 38A) with retention times 15 seconds apart. These were assumed to be the two isomers of phenanthrene dihydrodiol. Mass spectra of peak I and II are shown in Fig. 38B and 38C, respectively. They had the same mass spectra showing a M^+ at *m/z* 194 (base peak) and another major fragment ion at *m/z* 165 (M^+ -CHO). These molecular ions correspond to the two isomers of phenanthrol. However, results obtained after the neutral extract was derivatized with BSA or BTB indicated that peaks I and II actually consisted of two isomers of phenanthrene dihydrodiol. TMS derivatives of the neutral extract showed that one isomer eluted about 1.5 min later than the other (Fig. 39A). GC-MS analysis showed that the two peaks had the same mass spectral fragmentation with a M^+ at *m/z* 356 which indicated that two TMS groups reacted with the two hydroxyl groups of the phenanthrene dihydrodiol (Fig. 39 B & C).

BTB derivatives of the neutral extract showed two peaks which eluted about 1 min apart (Fig. 40A). GC-MS analysis showed that the two peaks had the same mass spectra with a M^+ at *m/z* 278 which showed fragment ions corresponding to stepwise degradation of the butyl side-chain (Fig. 40B & 40C). The BTB derivatized compounds with MW 278 indicated that one molecule of BTB reacted with two hydroxyl groups on the phenanthrene dihydrodiol. These results confirmed that *P. fluorescens* LP6a mutant D1 produced two isomers of phenanthrene dihydrodiol, presumably phenanthrene 1,2-dihydrodiol and phenanthrene 3,4-dihydrodiol.

The neutral extract of strain LP6a-D1 that contained 1,2- and 3,4-phenanthrene dihydrodiols and its TMS and BTB derivatives were then used as standards for coinjection with the neutral extract of *Rhodococcus* S1 and its TMS and BTB derivatives. Since the standards and the unknown compound X and their derivatives did not coelute, it confirmed that compound X was neither phenanthrene 1,2- nor 3,4-dihydrodiol.

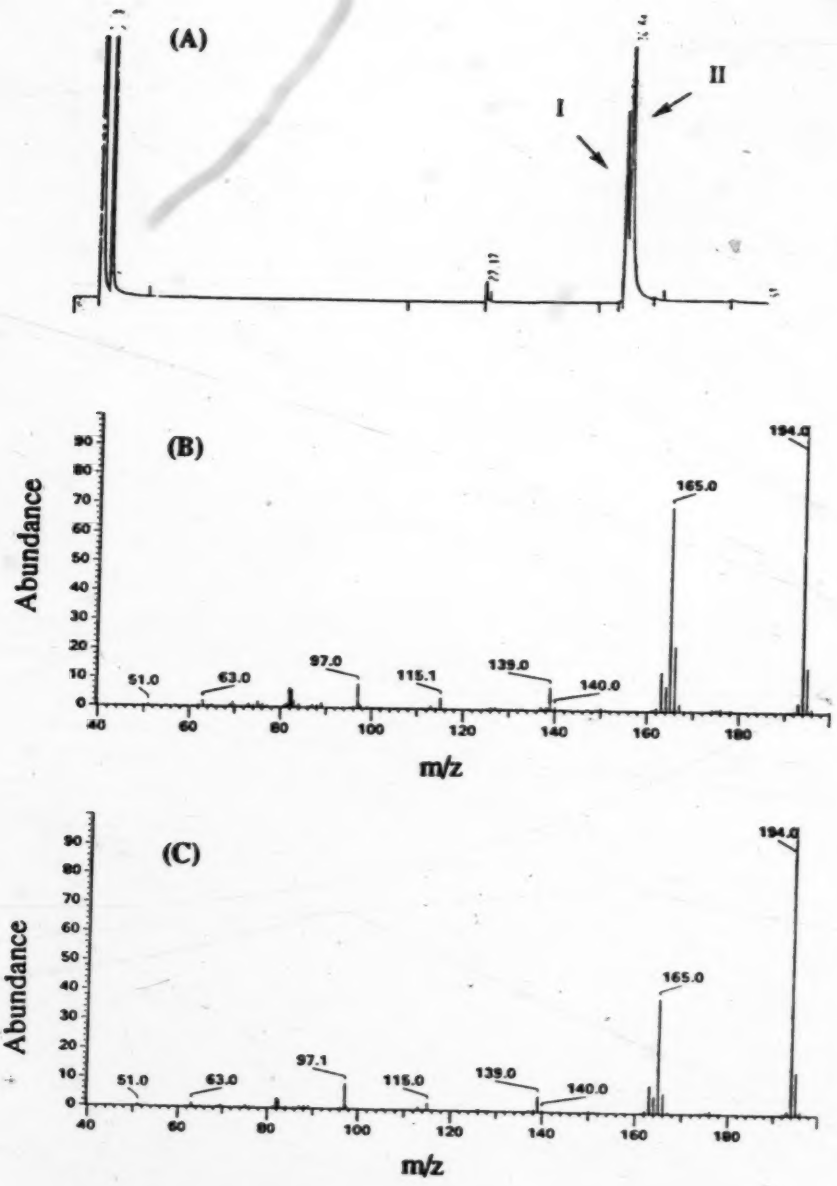


Fig. 38 (A) GC chromatogram of the neutral extract of *P. fluorescens* LP6a mutant D1 phenanthrene dihydrodiols showing peak I & II that had the same molecular weight. Mass spectra of peak I (B) and peak II (C).

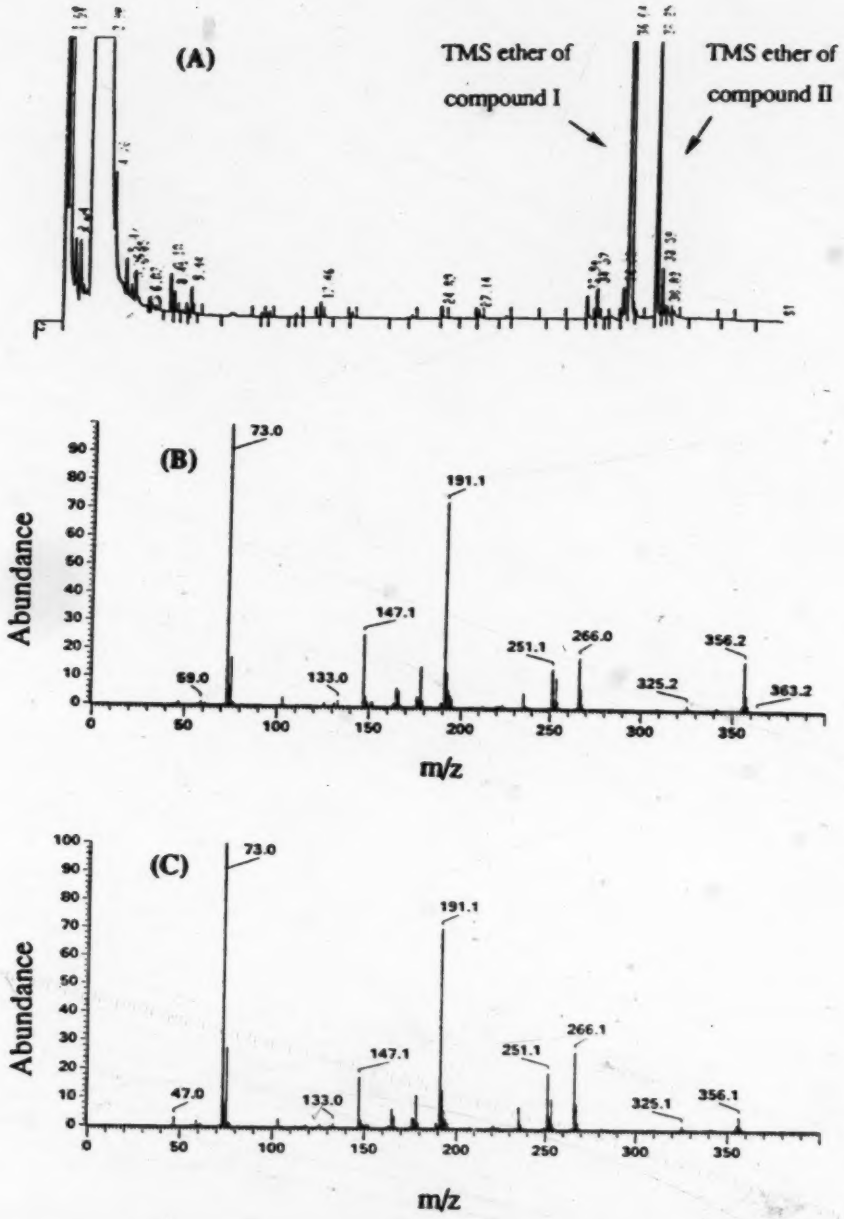


Fig. 39 (A) GC chromatogram of BSA-treated neutral extract of *P. fluorescens* LP6a mutant D1 showing the TMS ether of compound in peak I & II. Mass spectra of the TMS ether of compound I (B) and compound II (C).

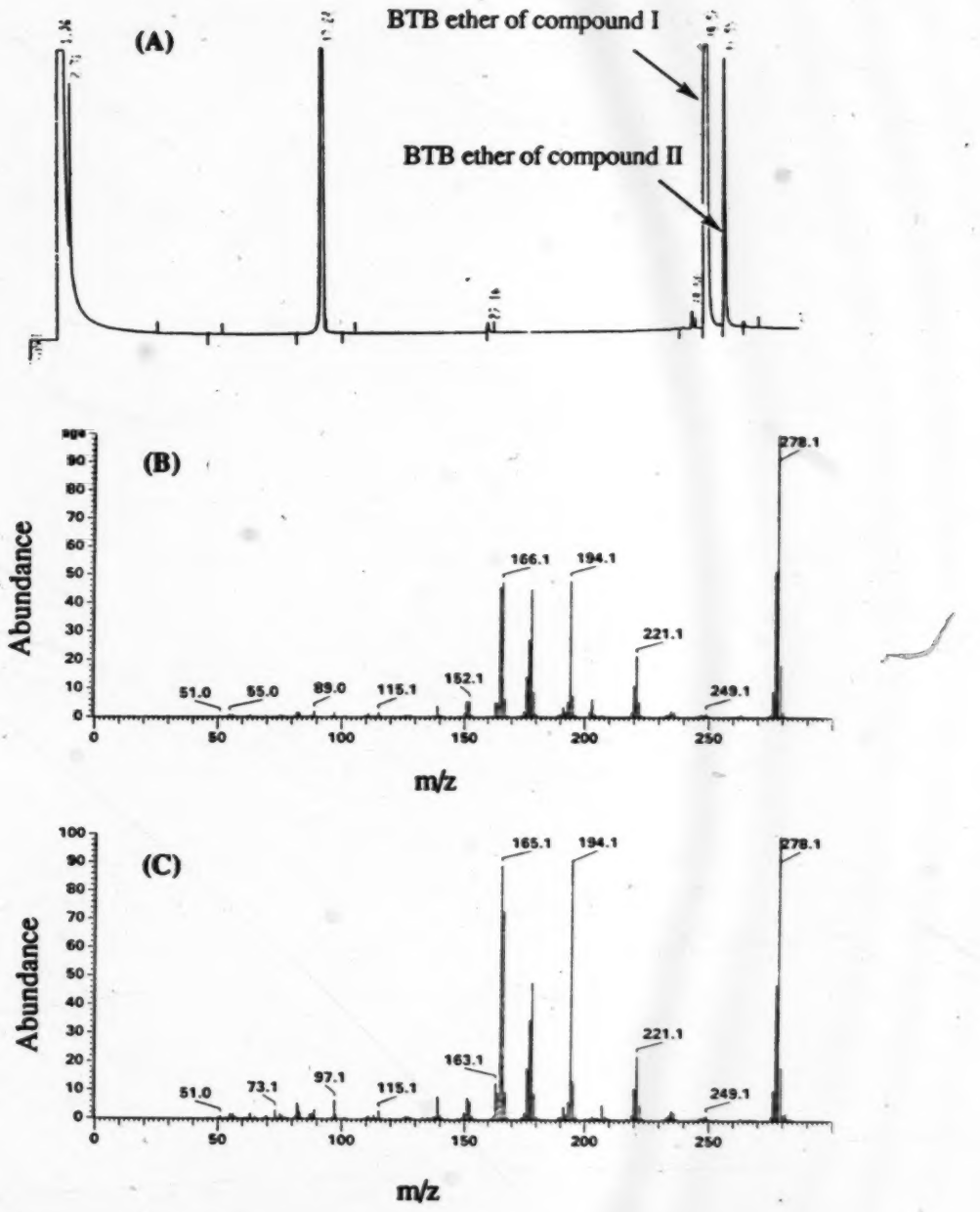


Fig. 40 (A) BTB derivatives of the neutral extract of *P. fluorescens* LP6a mutant D1 phenanthrene dihydrodiols and their mass spectra (B & C).

4.2.16 Production of *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene by chemical synthesis.

To determine if the *Rhodococcus* S1 phenanthrene dihydrodiol (compound X) was in the *cis*- form, as might be expected from the action of a dioxygenase, or the *trans*-form which might suggest a monooxygenase formation, *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene was chemically synthesized from phenanthrene 9,10-quinone. The product was analyzed by GC-FID and GC-MS. There were 2 peaks (I & II) which eluted only 13 seconds apart (Fig. 41A). GC-MS of peak I (Fig. 41B) showed clearly a M^+ at *m/z* 212 with the mass spectrum similar to phenanthrene dihydrodiol from *Rhodococcus* S1 (Fig. 19A) and anthracene dihydrodiol from *P. fluorescens* LP6a mutant D1 (Fig. 20A) with different abundance of molecular ions. Peak II had a very weak M^+ at *m/z* 212 (Fig. 41C). The fragment ion at *m/z* 181 was also much less abundant in peak II, while ions at *m/z* 194 and 165 had similar abundance. This suggested that the ion at *m/z* 181 in figure 41C might come from a M^+ at *m/z* 212 whereas the ion at m/z 165 came from the molecular ion at *m/z* 194. Since peak I was tailing and overlapping peak II, it was more likely that a tiny M^+ at *m/z* 212 shown in Fig. 41C was contributed by peak I and that the ion at *m/z* 194 was the actual molecular ion of peak II . This M^+ at *m/z* 194 was more likely to be 9-phenanthrol, a product of dehydration of the M^+ at *m/z* 212. The next abundant ion at *m/z* 165 indicated the loss of 29 mass unit (CHO) of M^+ at m/z 194 which normally occurred in mass spectral fragmentation of aromatic alcohol.

The extract was also derivatized with BSA. It yielded only one peak that had a M^+ at *m/z* 356 (Fig. 42A) and mass spectrum similar to that of the TMS derivative of phenanthrene 9,10-dihydrodiol (Fig. 19 A). This result indicated that two groups of TMS reacted with two hydroxyl groups on the compound to form the TMS ether of phenanthrene dihydrodiol. BTB derivatization of the extract gave a peak that had a M^+ at *m/z* 278 (Fig. 42B) which showed a similar mass spectrum to that of the BTB derivative of

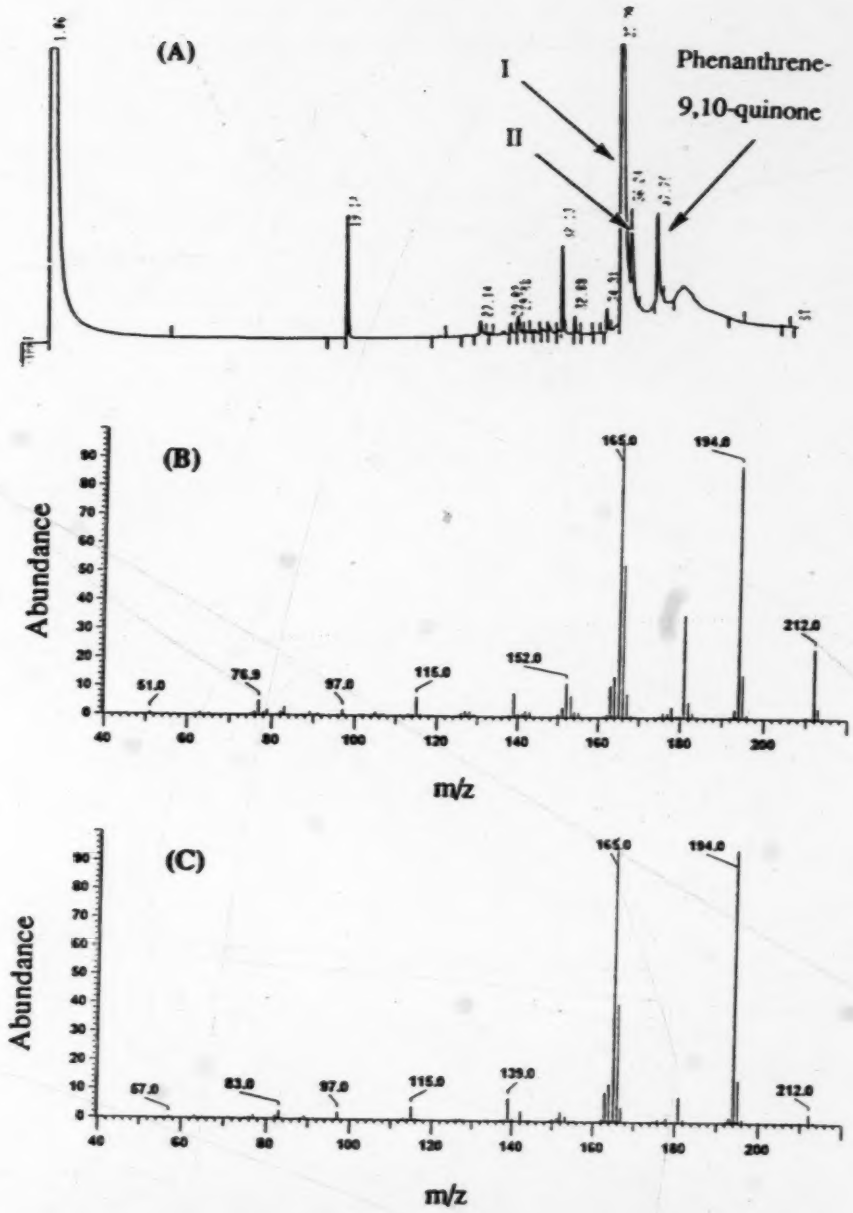


Fig. 41 (A) GC chromatogram of the EtAc extract containing chemically synthesized *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene showing compound I and II together with their mass spectra (B & C) respectively.

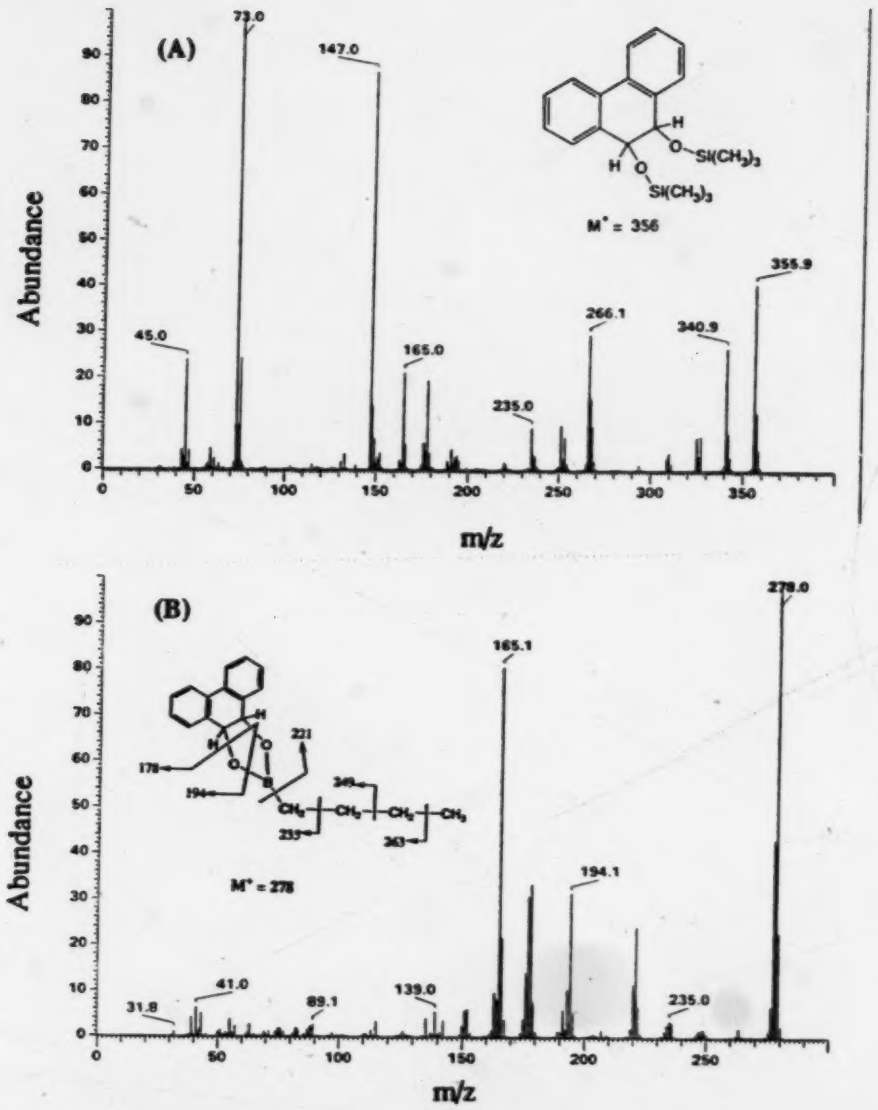


Fig. 42 Mass spectra of TMS derivative (A) and BTB derivative (B) of compound I in the extract of chemically synthesized *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene.

phenanthrene 9,10-dihydrodiol from *Rhodococcus* S1 (Fig. 36).

By coinjection, it was shown that chemically synthesized *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene and its TMS derivative coeluted with the phenanthrene 9,10-dihydrodiol from *Rhodococcus* S1 and its TMS derivative, respectively. This suggested that phenanthrene 9,10-dihydrodiol produced from *Rhodococcus* S1 when grown on anthracene was likely to be *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene.

4.3 Cometabolism of various PAHs by *Rhodococcus* S1 grown on anthracene.

Since phenanthrene 9,10-dihydrodiol was found as a cometabolite from *Rhodococcus* S1, other potential substrate PAHs were also investigated. *Rhodococcus* S1 was grown on anthracene in the presence of one of the following PAHs: naphthalene, biphenyl, acenaphthene, fluorene and fluoranthene (each at 0.05% final concentration); and pyrene, chrysene, benzanthracene, naphthacene, and 2-methylphenanthrene (each at 0.02% final concentration). Sterile controls of the individual PAH were also incubated. Oxidized products of these PAHs were observed from all PAHs tested except benzanthracene, naphthacene, and 2-methylphenanthrene.

4.3.1 Cometabolism of naphthalene.

Analyses by GC of the neutral extract of *Rhodococcus* S1 medium grown on anthracene in the presence of naphthalene yielded 5 metabolite peaks designated as I, II, III, IV and V. Metabolite I was readily identified as 1-naphthol. Its mass spectrum had a M^+ at *m/z* 144 as a base peak (Fig. 43A). The next abundant fragment ion at *m/z* 115 indicated the loss of 29 mass unit (CHO) from the M^+ at *m/z* 144 which normally occurs in mass spectra of aromatic alcohols (Silverstein *et al.* 1991). The mass spectrum of authentic 1-naphthol is shown in Fig. 43B. Coinjection of the neutral extract with the authentic 1-

naphthol revealed that metabolite I coeluted with the authentic compound. The TMS derivative of the neutral extract showed a peak that had a M^+ at *m/z* 216 indicating that a molecule of TMS reacted with the single hydroxyl group of the metabolite (Fig. 44A). Its mass spectrum had a similar fragmentation pattern to that of the TMS ether of authentic 1-naphthol (Fig. 44B). As expected, the TMS ether of metabolite I coeluted with the TMS ether of authentic 1-naphthol.

The next metabolite found (II) was tentatively identified as naphthalene dihydrodiol. Its mass spectrum (Fig. 45A) had a M^+ at *m/z* 162 with the next fragment ion at *m/z* 144 indicating the loss of 18 mass unit (M^+-H_2O) which is normally observed in dihydrodiols as reported for phenanthrene *trans*-1,2-dihydrodiol produced by *Cunninghamela elegans* (Cerniglia and Yang 1984) and as shown in this study for anthracene *cis*-1,2-dihydrodiol produced from *P. fluorescens* LP6a mutant D1 (see section 4.2.8). Analysis of the TMS-derivatized extract revealed a M^+ at *m/z* 306 consistent with the incorporation of two molecules of trimethylsilane into a molecule of naphthalene dihydrodiol (Fig. 45B).

The mass spectrum of metabolite III showed a M^+ at *m/z* 160 with a fragment ion at *m/z* 131 (M^+ -CHO) (Fig. 46A). This compound had a fragmentation pattern similar to that of 1,4-dihydroxynaphthalene found in the National Institute of Standards and Technology (NIST) data base library search (Fig. 46B). Only some isomers of dihydroxynaphthalene were commercially available i.e. 1,2-; 2,3-; 1,3-; 1,5-; 1,6-; and 2,7- but not the 1,4-isomer. Coinjection of the neutral extract and the available standards indicated that the dihydroxynaphthalene from the bacterium was not any of the available isomers mentioned above. Thus, metabolite III was likely 1,4-dihydroxynaphthalene, but an authentic standard was not available to prove this identification. After BSA derivatization, GC-MS analysis of the neutral extract yielded a peak that had a M^+ at *m/z*

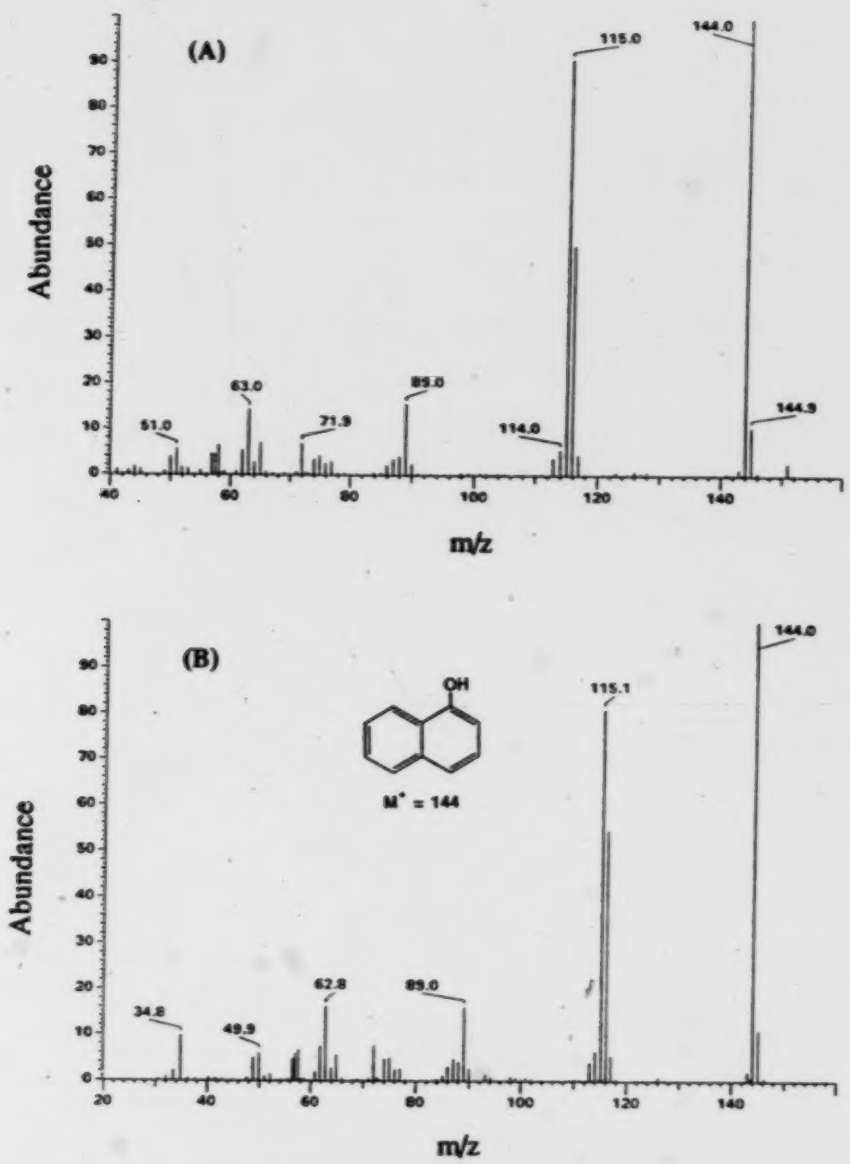


Fig. 43 Mass spectra of metabolite I obtained from *Rhodococcus* S1 grown on anthracene in the presence of naphthalene (A) and the authentic 1-naphthol (B).

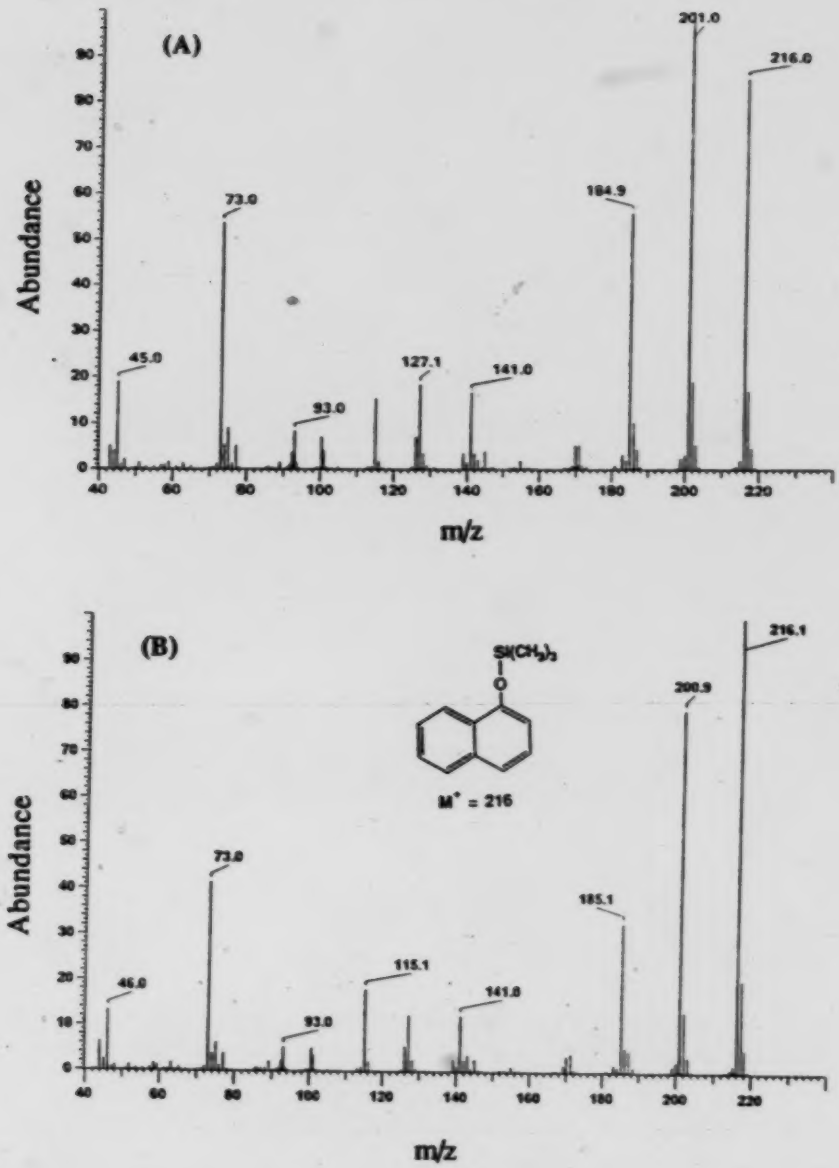


Fig. 44 Mass spectra of TMS derivatives of metabolite I from *Rhodococcus* S1 (A) and the authentic 1-naphthol (B).

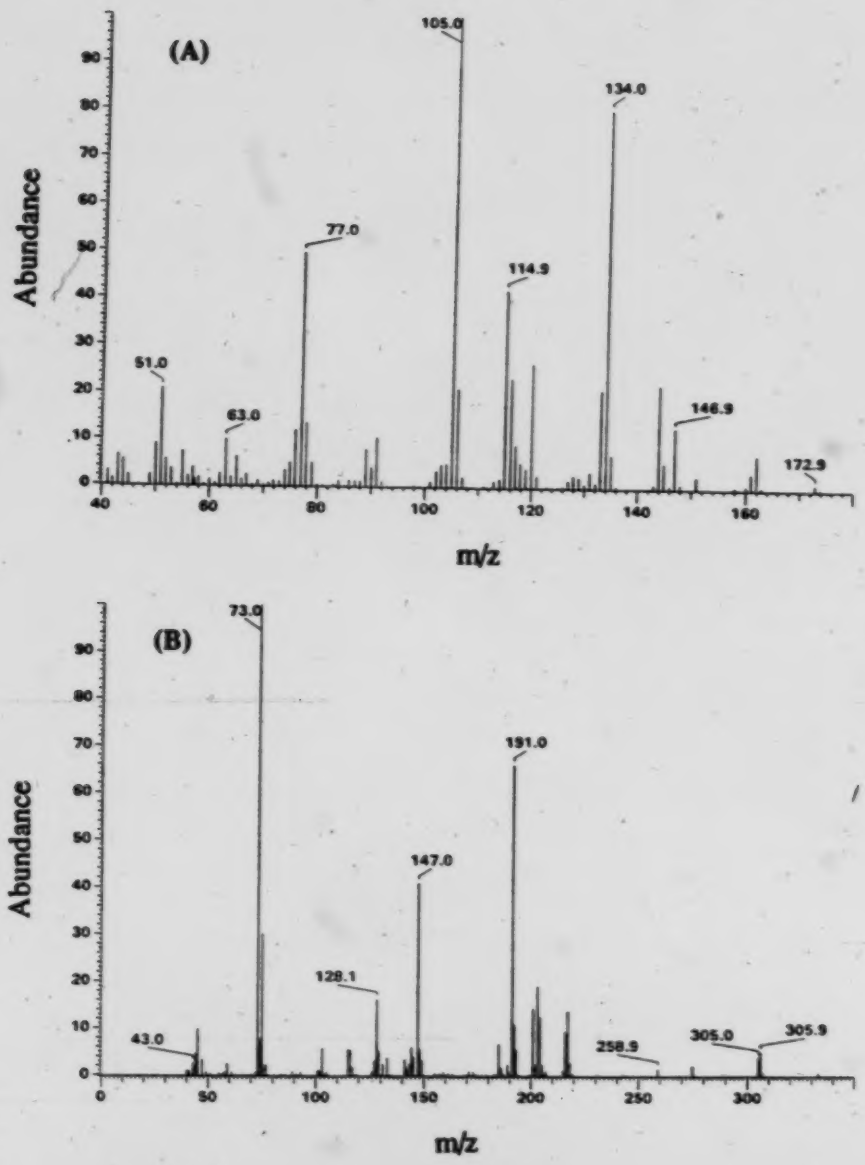


Fig. 45 Mass spectra of the metabolite II (A) and its TMS derivative (B) obtained from *Rhodococcus* S1 grown on anthracene in the presence of naphthalene which corresponded to naphthalene dihydrodiol.

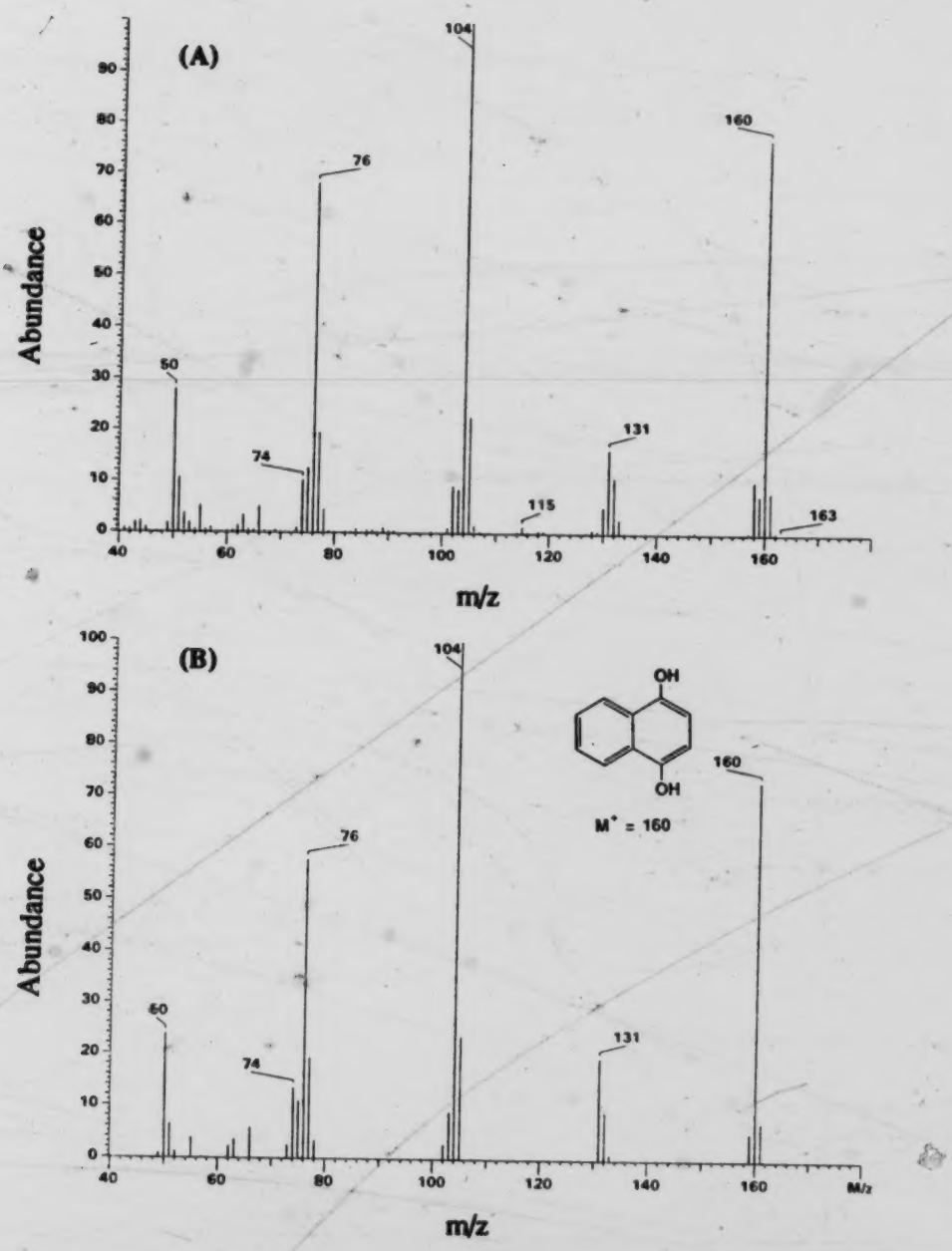


Fig. 46 Mass spectra of metabolite III, tentatively identified as dihydroxynaphthalene, found in the neutral extract of *Rhodococcus* S1 (A) and 1,4-dihydroxynaphthalene published in the NIST data base library (B).

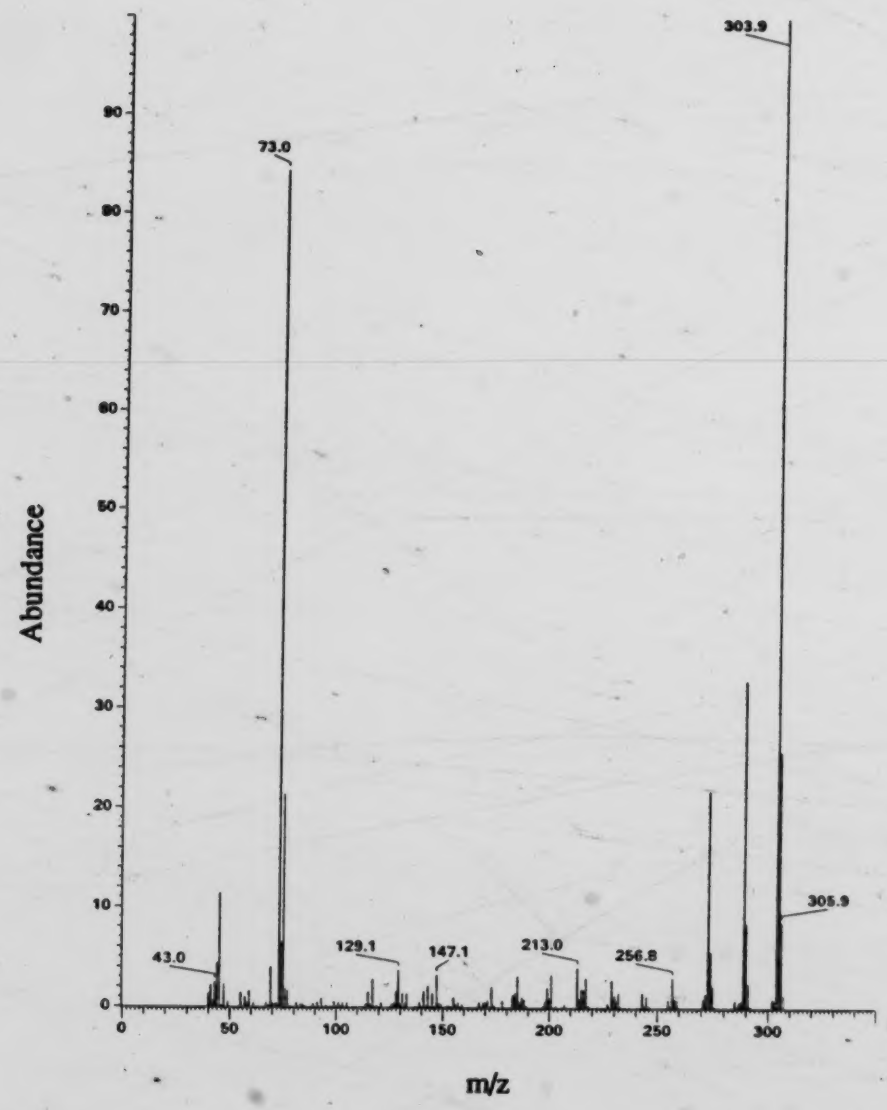


Fig. 47 The mass spectrum of TMS derivative of metabolite III in the neutral extract of *Rhodococcus* S1 which was consistent with dihydroxynaphthalene.

304 (Fig. 47) which corresponded to a molecule of dihydroxynaphthalene that had incorporated two TMS groups. This suggested that dihydroxynaphthalene found in the neutral extract of *Rhodococcus* S1 was likely 1,4-dihydroxynaphthalene.

Among the naphthalene metabolites found in the neutral extract of *Rhodococcus* S1 were other two compounds (IV and V) that corresponded to naphthoquinones. On GC analysis they eluted about 1 min apart. GC-MS analyses revealed that compound IV (Fig. 48A) and compound V (Fig. 48B) had a M^+ at *m/z* 158 but with different fragmentation patterns. Coinjection of the neutral extract with the commercially available 1,2- and 1,4-naphthoquinones revealed that compound IV coeluted with authentic 1,4-naphthoquinone. Mass spectra of the commercially available 1,4-naphthoquinone and 1,2-naphthoquinone are shown in Fig. 48C & 48D respectively. As expected, the mass spectrum of compound IV matched that of authentic 1,4-naphthoquinone. This indicated that *Rhodococcus* S1 produced two isomers of naphthoquinone, one of which was identified as 1,4-naphthoquinone. The exact structure of the other isomer of naphthoquinone is unknown as yet since authentic standards of the other isomers are not commercially available.

4.3.2 Cometabolism of biphenyl.

The oxidized products of biphenyl found from *Rhodococcus* S1 were tentatively identified as a dihydroxybiphenyl and 2 isomers of hydroxybiphenyl. During coinjection with the authentic compounds, one isomer of hydroxybiphenyl coeluted with 2-hydroxybiphenyl and the other isomer coeluted with 4-hydroxybiphenyl. Fig. 49A and 49B show the mass spectra of hydroxybiphenyl found in *Rhodococcus* S1 culture extract and the authentic 2-hydroxybiphenyl, respectively. The mass spectrum of 4-hydroxybiphenyl from strain S1 and the authentic 4-hydroxybiphenyl are shown in Fig. 50 A and 50B, respectively. The mass spectra of hydroxybiphenyls from *Rhodococcus* S1

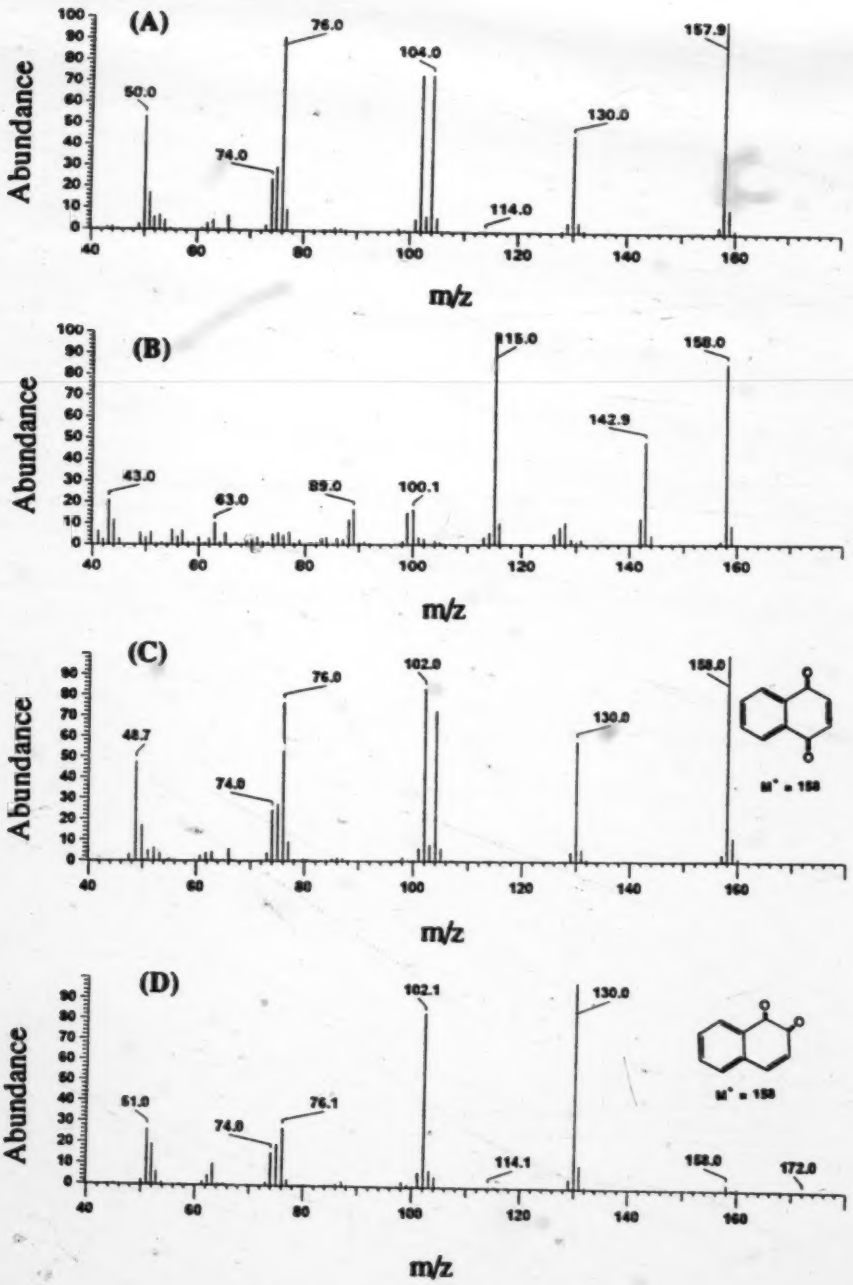


Fig. 48 Mass spectra of metabolite IV (A), metabolite V (B), the authentic 1,4-naphthoquinone (C), and 1,2-naphthoquinone (D).

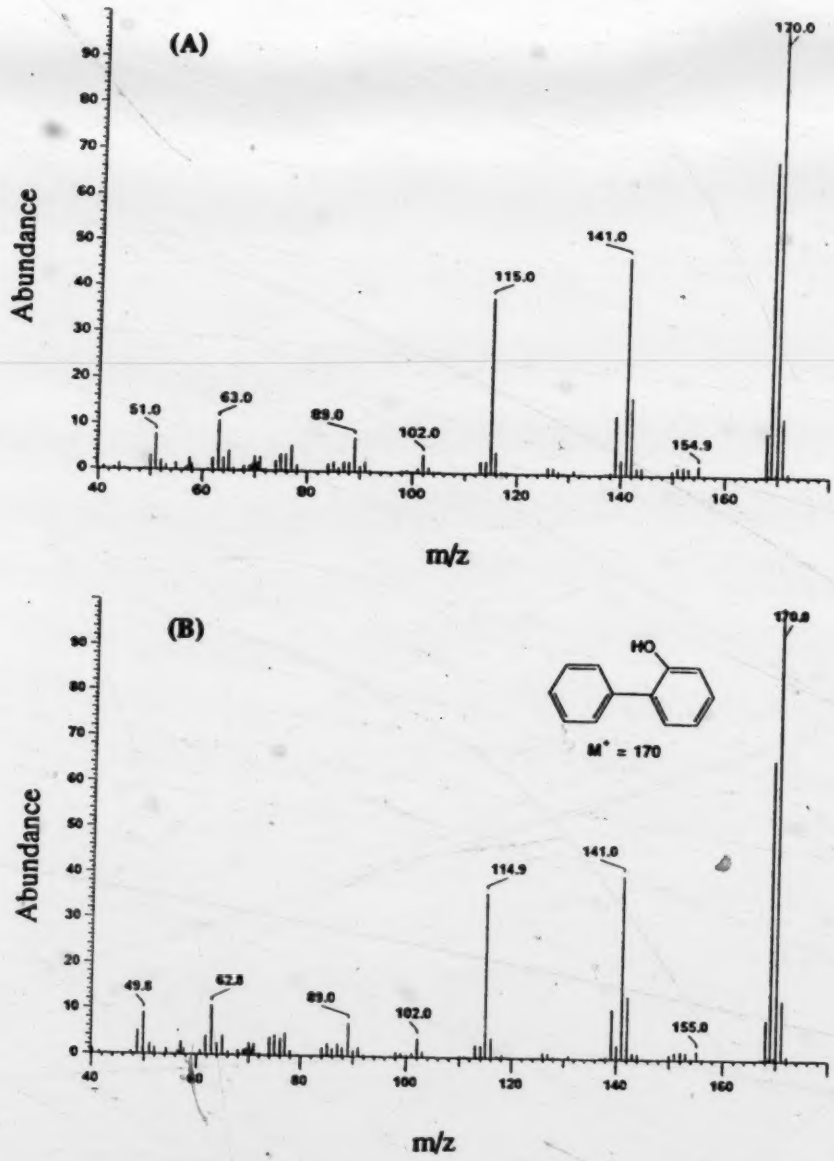


Fig. 49 Mass spectra of the 2-hydroxybiphenyl. (A) The 2-hydroxybiphenyl produced by *Rhodococcus* S1. (B) Authentic 2-hydroxybiphenyl.

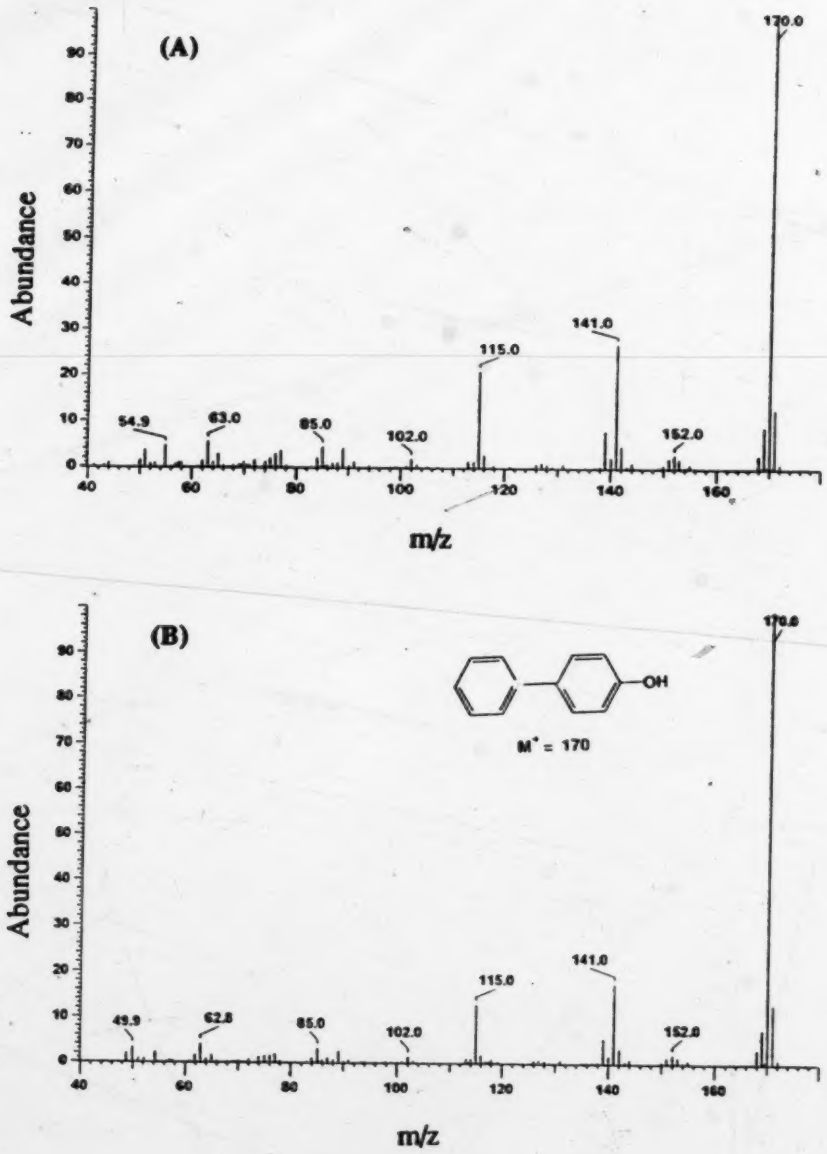


Fig. 50 Mass spectra of the 4-hydroxybiphenyl. (A) The 4-hydroxybiphenyl produced by *Rhodoccoccus* S1. (B) Authentic 4-hydroxybiphenyl.

showed a M^+ at *m/z* 170 as the base peak. The next abundant fragment ion at *m/z* 141 indicated a loss of 29 mass units (M^+ -CHO) which is typical of aromatic alcohols (Silverstein *et al.* 1991). The mass spectra of the TMS ether of the 2-hydroxybiphenyl (Fig.51A) and 4-hydroxybiphenyl (Fig. 52A) from *Rhodococcus* S1 were consistent with those of the authentic 2-hydroxybiphenyl (Fig. 51B) and 4-hydroxybiphenyl (Fig. 52B), respectively. The TMS ethers of the 2- and 4-hydroxybiphenyl had the similar mass spectral fragmentation patterns with different abundance of the major ions.

The dihydroxybiphenyl metabolite obtained from *Rhodococcus* S1 gave a mass spectrum (Fig. 53A) that had a M^+ at *m/z* 186 and the next abundant ion at *m/z* 157 (M^+ -CHO). The spectrum of its TMS ether is shown in Fig. 53B. Coinjection of the underivatized extract from *Rhodococcus* S1 with the commercially available 2,2'-dihydroxybiphenyl and 4,4'-dihydroxybiphenyl indicated that dihydroxybiphenyl from *Rhodococcus* S1 was not either the 2,2'- or 4,4'-isomer.

These results confirmed that *Rhodococcus* S1 when grew on anthracene could transform biphenyl into 2- and 4-hydroxybiphenyl and a dihydroxybiphenyl.

4.3.3 Cometabolism of acenaphthene.

GC analysis of the neutral extract of anthracene-grown *Rhodococcus* S1 incubated with acenaphthene revealed two metabolite peaks (I and II) that were potentially oxidized products of acenaphthene. The mass spectrum of peak I had a M^+ at *m/z* 170 (base peak) and a fragment ion at *m/z* 141 (M^+ -CHO) which corresponded to acenaphthenol (Fig. 54A). Mass spectrum of peak II showed a M^+ at *m/z* 168 and a fragment ion at *m/z* 140 (M^+-CO) as the base peak (Fig. 54B) which was consistent with acenaphthenone. GC-MS analysis of the BSA derivatized neutral extract revealed two TMS derivatives which eluted approximately 6.5 min apart. Both of them showed a M^+ at *m/z* 242 which indicated that a single molecule of TMS had reacted with a hydroxy group of acenaphthenol. Each

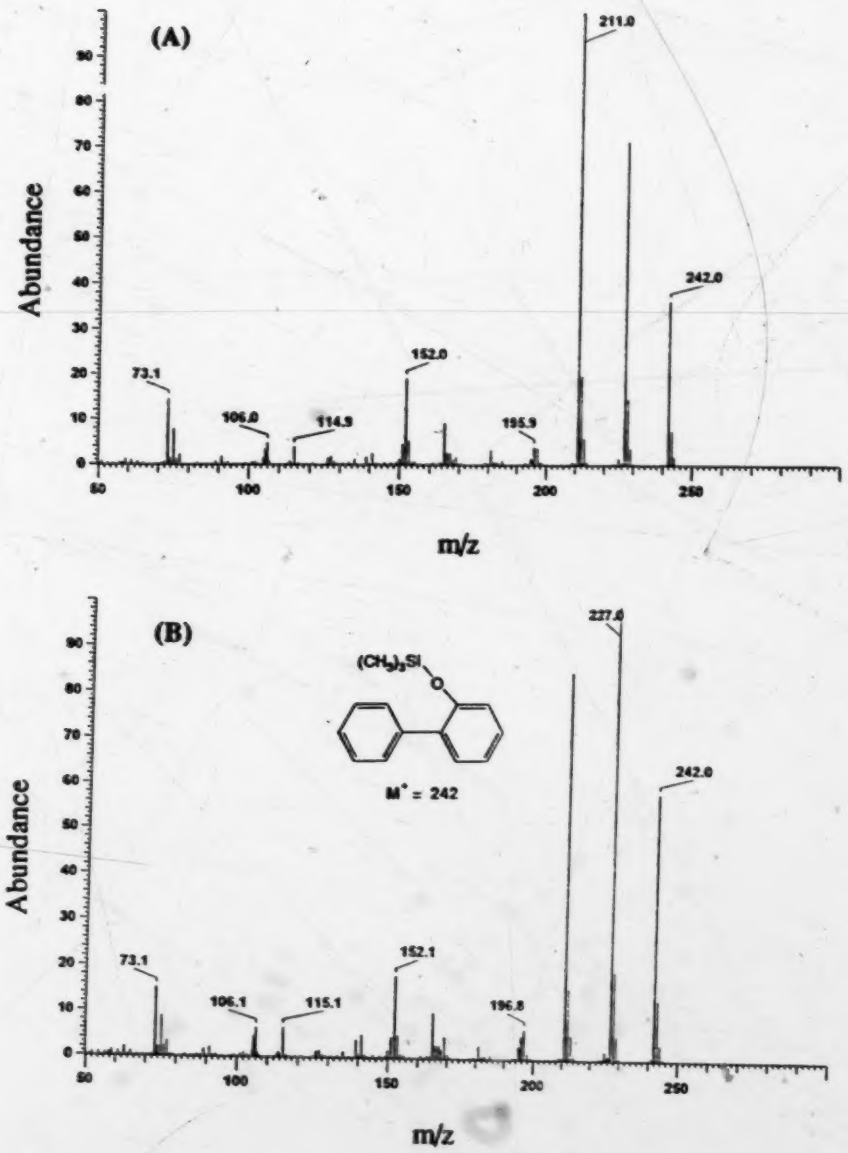


Fig. 51 Mass spectra of the TMS ether of 2-hydroxybipheyl produced by *Rhodococcus* S1 (A) and the authentic 2-hydroxybiphenyl.

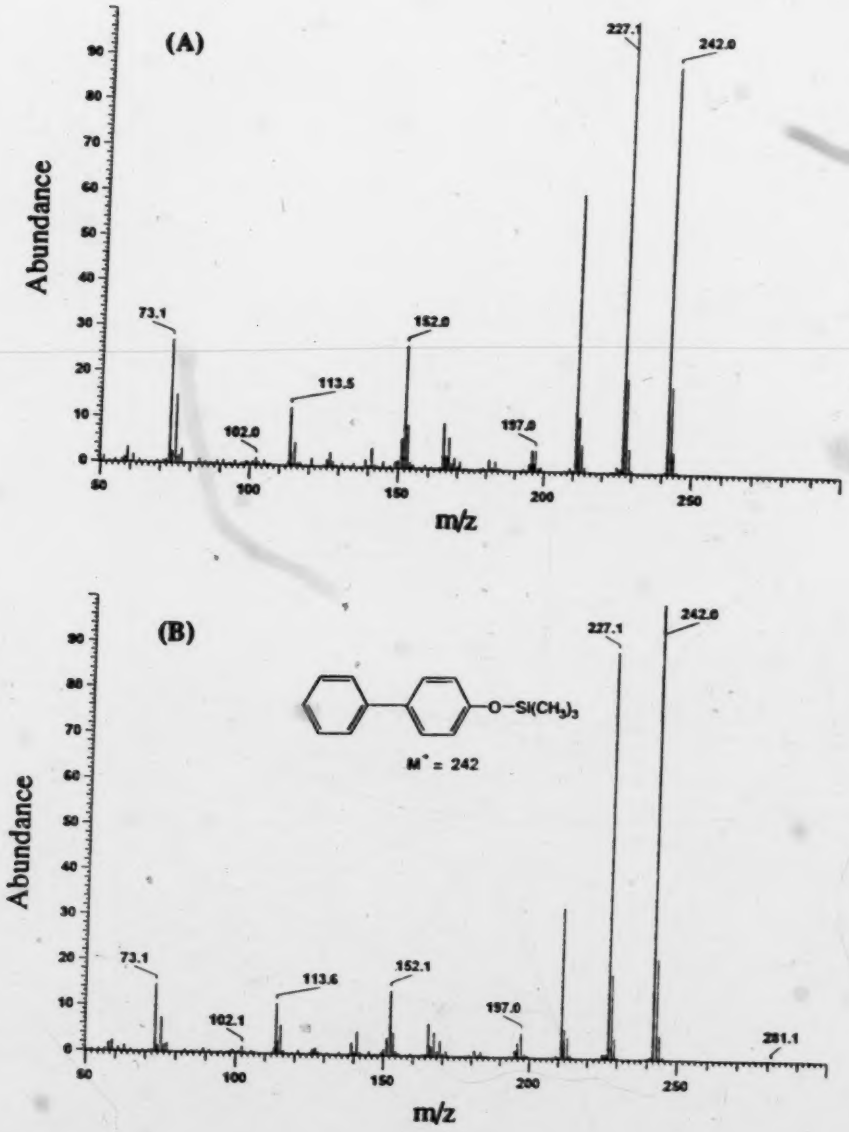


Fig. 52 Mass spectra of the TMS ether of 4-hydroxybiphenyl produced by *Rhodococcus* S1 (A) and the authentic 4-hydroxybiphenyl.

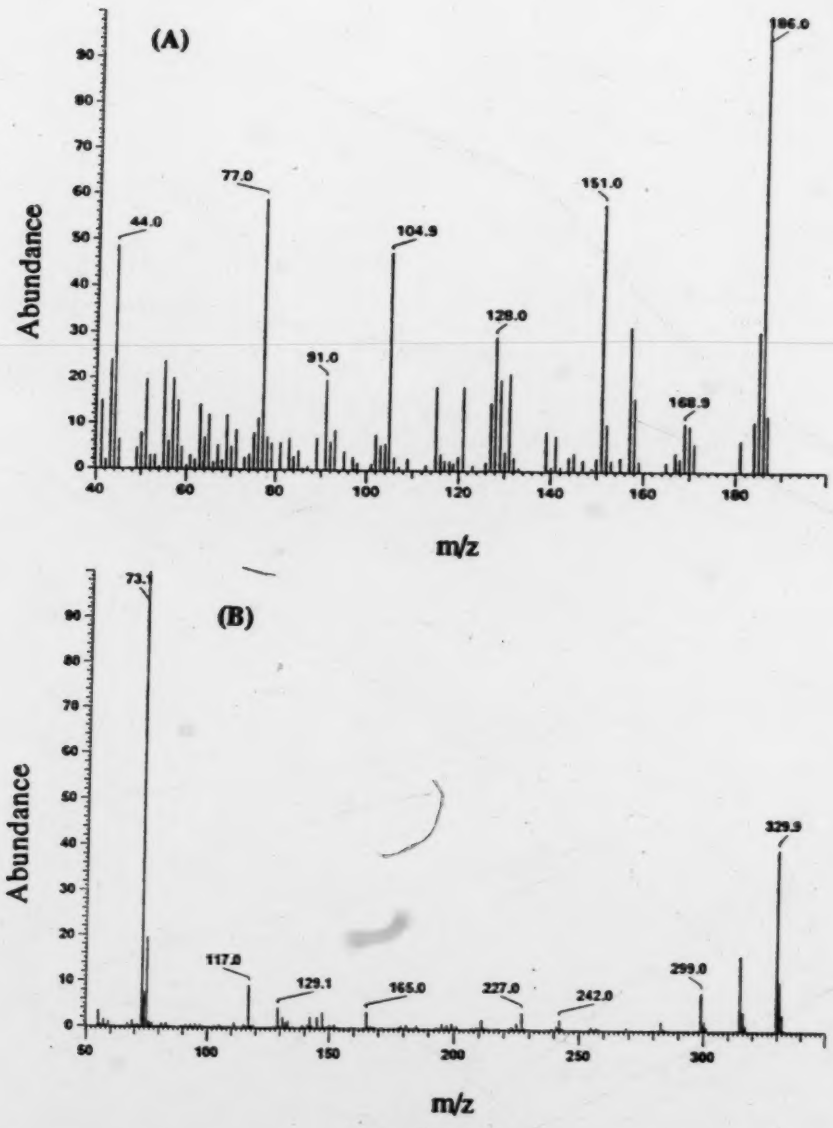


Fig. 53 Mass spectra of a compound produced by *Rhodococcus* S1 which was tentatively identified as dihydroxybiphenyl (A) and its TMS derivative (B).

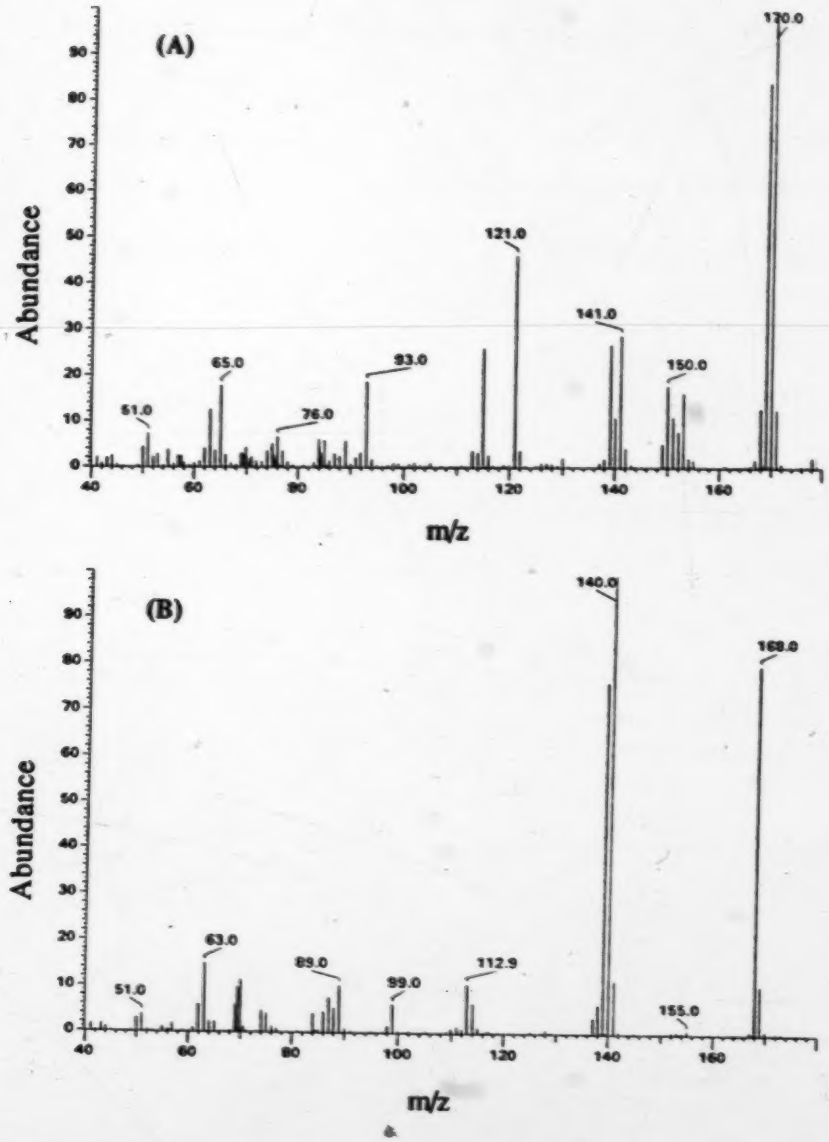


Fig. 54 Mass spectra of the metabolite I (A) and metabolite II (B) produced in a spent medium of *Rhodococcus* S1 when grown in the presence of acenaphthene which were tentatively identified as acenaphthenol and acenaphthenone, respectively.

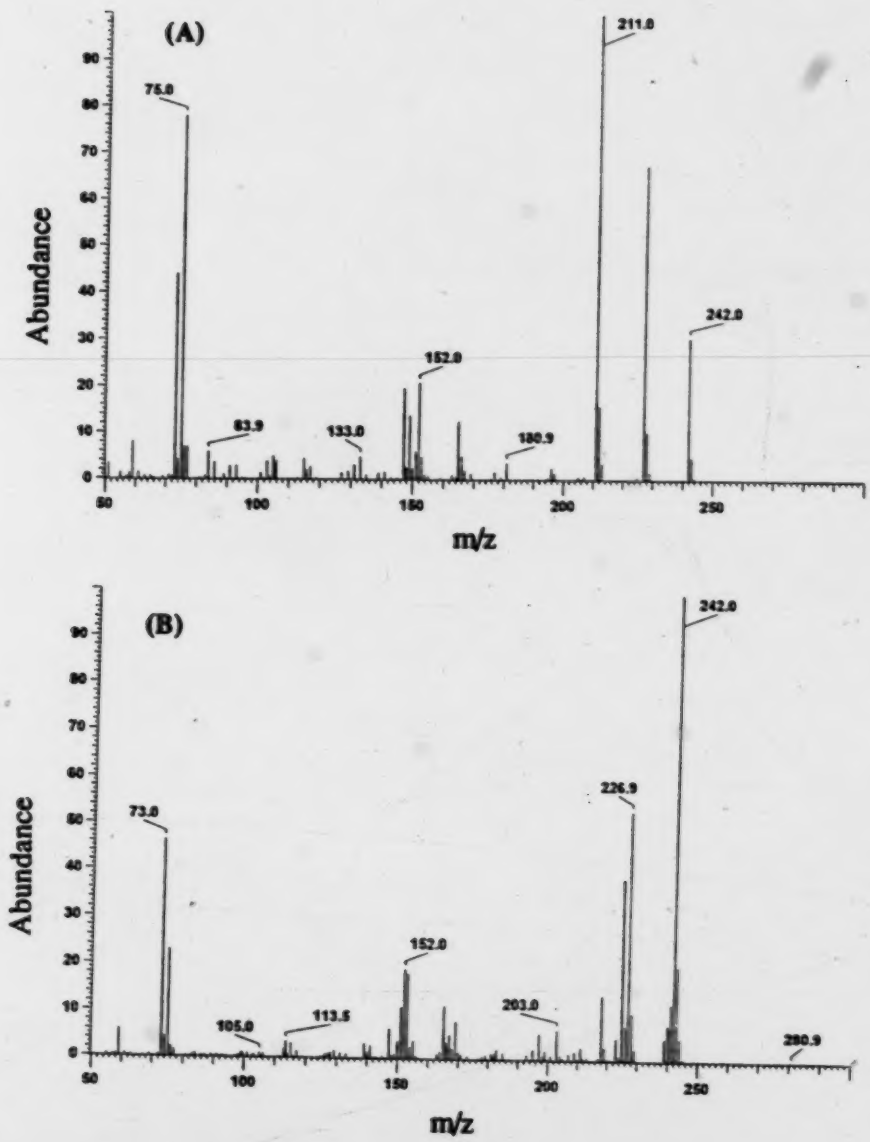


Fig. 55 Mass spectra of TMS derivative of metabolites that corresponded to acenaphthenol produced by *Rhodococcus* S1 grown in the presence of acenaphthene.

compound showed a similar mass spectrum (Fig. 55A & 55B) with a different ion abundance. It was not surprising to find two peaks of TMS ether of acenaphthenol after BSA derivatization, since acenaphthenone might undergo keto-enol tautomerization and subsequently react with BSA.

4.3.4 Cometabolism of fluorene.

The neutral extract of *Rhodococcus* S1 grown on anthracene in the presence of fluorene showed 2 peaks by GC analysis that corresponded to fluorenol. The major peak which was the largest, I, had a retention time of 29.12 min while the minor peak, II, had a retention time of 29.43 min. The mass spectra of metabolites I and II are shown in Fig. 56A and 56B, respectively, and are similar to that of the authentic 2-fluorenol (Fig. 56C). The mass spectra of TMS ethers of metabolites I, II, and authentic 2-fluorenol are shown in Fig. 57A, 57B, and 57C respectively. They all had similar fragmentation patterns with different ion abundances.

Coinjection of the commercially available 2- and 9-fluorenol with the neutral extract revealed that metabolite II coeluted with the 2-fluorenol. Likewise, the TMS ether of metabolite II also coeluted with TMS ether of 2-fluorenol. It was concluded that *Rhodococcus* S1 metabolized fluorene to two isomers of fluorenol; one of which was identified as 2-fluorenol. The position of the hydroxy group of the more abundant fluorenol is still unknown.

Even though fluorenols were the only oxidized products of fluorene found in the underivatized neutral extract, a TMS derivative of a compound corresponding to a dihydroxyfluorene was also observed in the extract after derivatization with BSA. Its mass spectrum showed a M^+ at *m/z* 342 (Fig. 58) consistent with two molecules of TMS reacting with the two hydroxyl groups of dihydroxyfluorene.

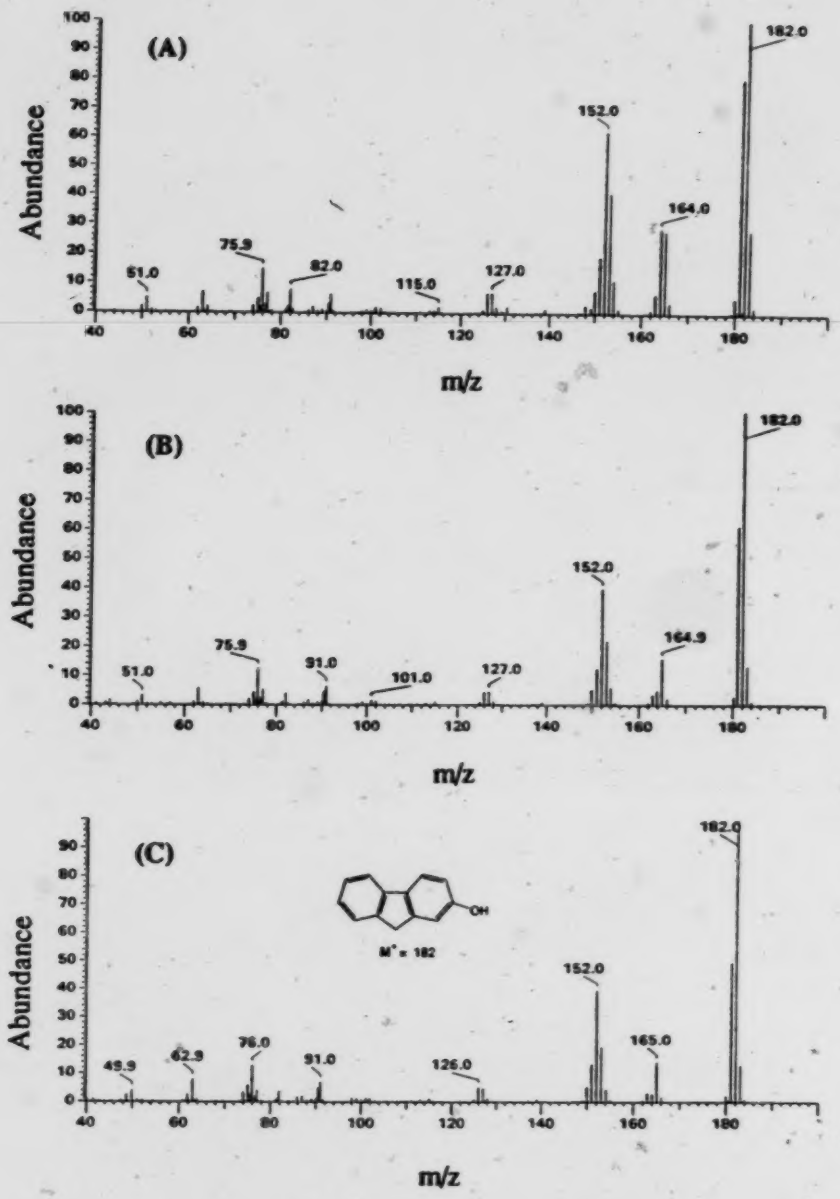


Fig. 56 Mass spectra of metabolite I (A), metabolite II (B) from *Rhodococcus* S1 grown on anthracene in the presence of fluorene and the authentic 2-fluorenol (C).

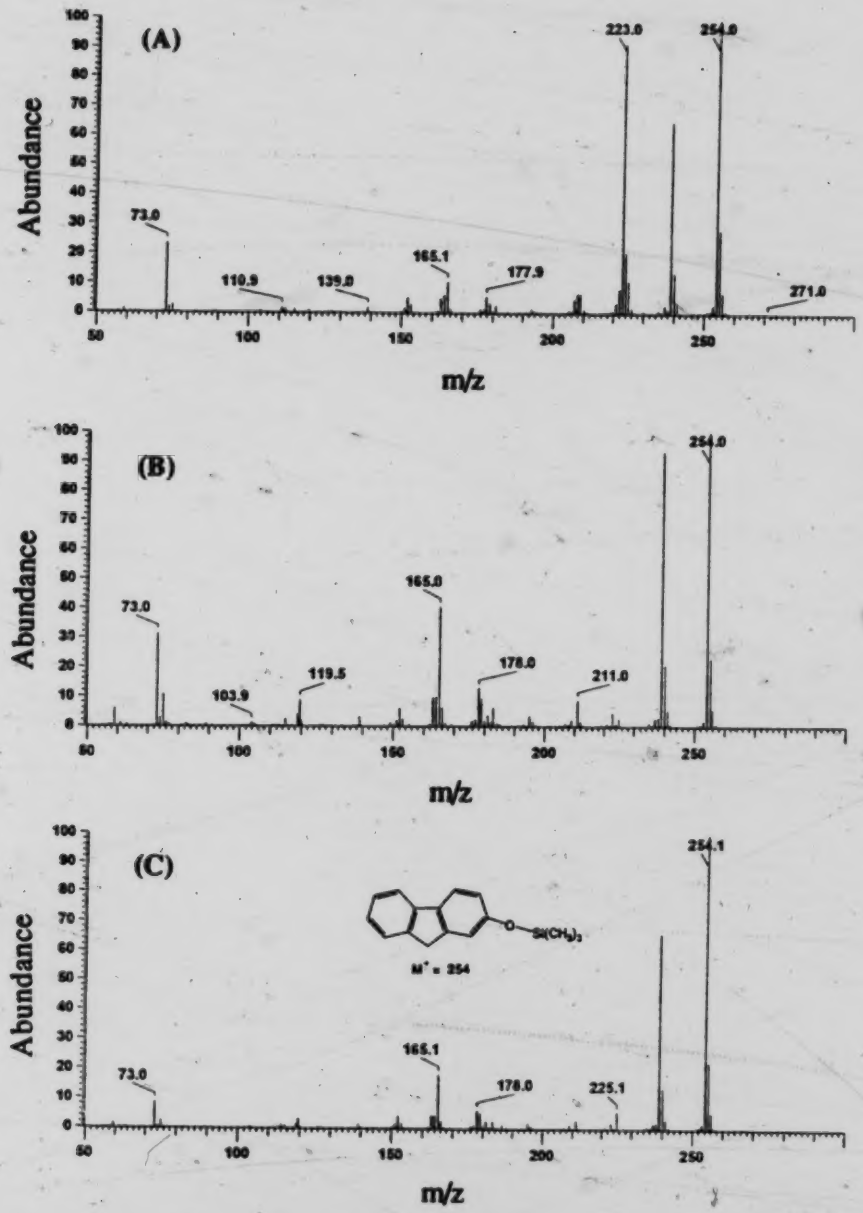


Fig. 57 Mass spectra of TMS derivatives of metabolite I (A), metabolite II (B) from *Rhodococcus* S1 grown on anthracene in the presence of fluorene and the authentic 2-fluorenol (C).

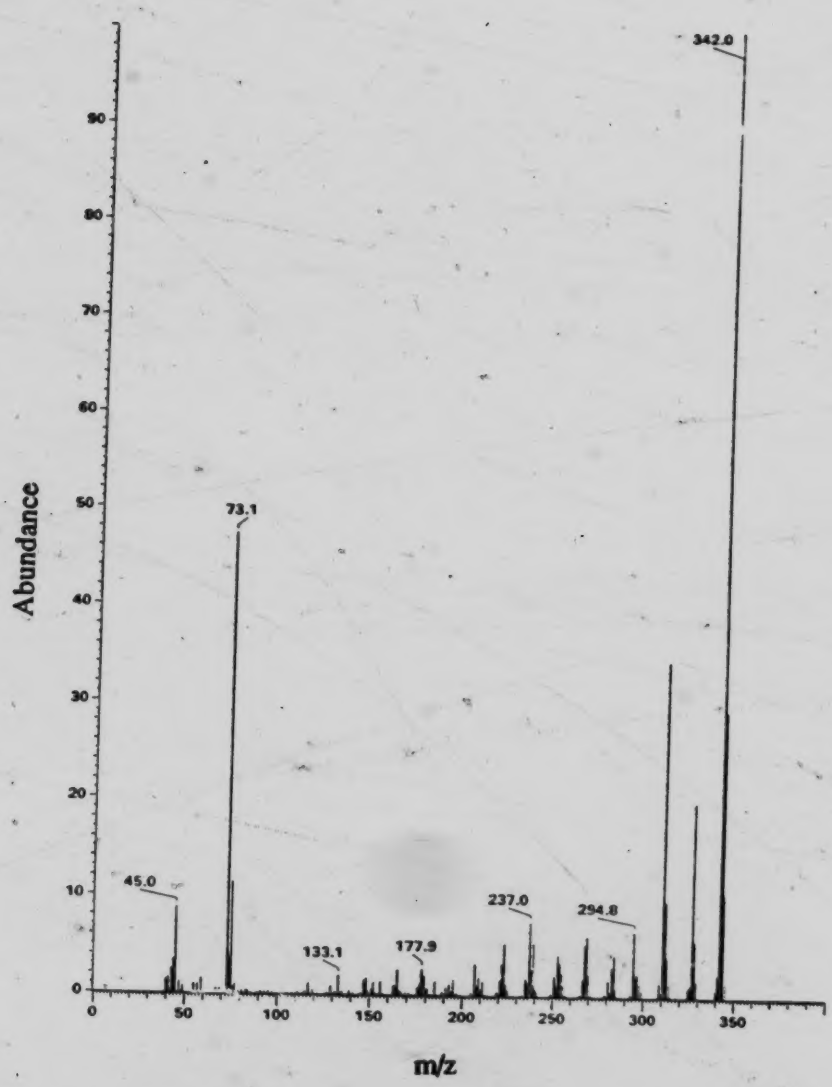


Fig. 58 The mass spectrum of TMS derivative of a compound found in the neutral extract of *Rhodococcus* S1 which was consistent with dihydroxyfluorene.

4.3.5 Cometabolism of fluoranthene.

GC analysis of the neutral extract of *Rhodococcus* S1 grown on anthracene in the presence of fluoranthene revealed 2 peaks (the major designated as I; the minor as II) that corresponded to fluoranthene dihydrodiol. In addition, another two peaks (the major as III; the minor, IV) were consistent with hydroxyfluoranthene. The fragmentation pattern of compound I (Fig. 59A) & compound II (Fig. 59B) were similar with different ion abundances. They both had a M^+ at *m/z* 236 and the next fragment ion at *m/z* 218 (M^+ - H_2O) indicating the loss of 18 mass unit which is normally observed in dihydrodiols (see explanation in section 4.3.1). Mass spectra of compound III (Fig. 60A) & compound IV (Fig. 60B) had a M^+ at *m/z* 218 (base peak) and the next abundant ion at *m/z* 189 (M^+ -CHO) again, typical of aromatic alcohols (Silverstein *et al.* 1991).

After the neutral extract was derivatized with BSA, it yielded 3 peaks of interest. There was only one peak that showed a mass spectrum with a M^+ at *m/z* 380 indicating two TMS groups had incorporated into a molecule of fluoranthene dihydrodiol (Fig. 61A). However, there was another peak that showed a mass spectrum with a M^+ at *m/z* 378 suggesting that two TMS groups had incorporated into a molecule of dihydroxyfluoranthene (Fig. 61B). The third peak showed a mass spectrum that had a M^+ at *m/z* 290 indicating one TMS group had incorporated into the molecule of hydroxyfluoranthene (Fig. 61C).

4.3.6 Cometabolism of pyrene.

Neutral extract of *Rhodococcus* S1 grown on anthracene in the presence of pyrene revealed one metabolite by GC analysis that corresponded to pyrene dihydrodiol. Mass

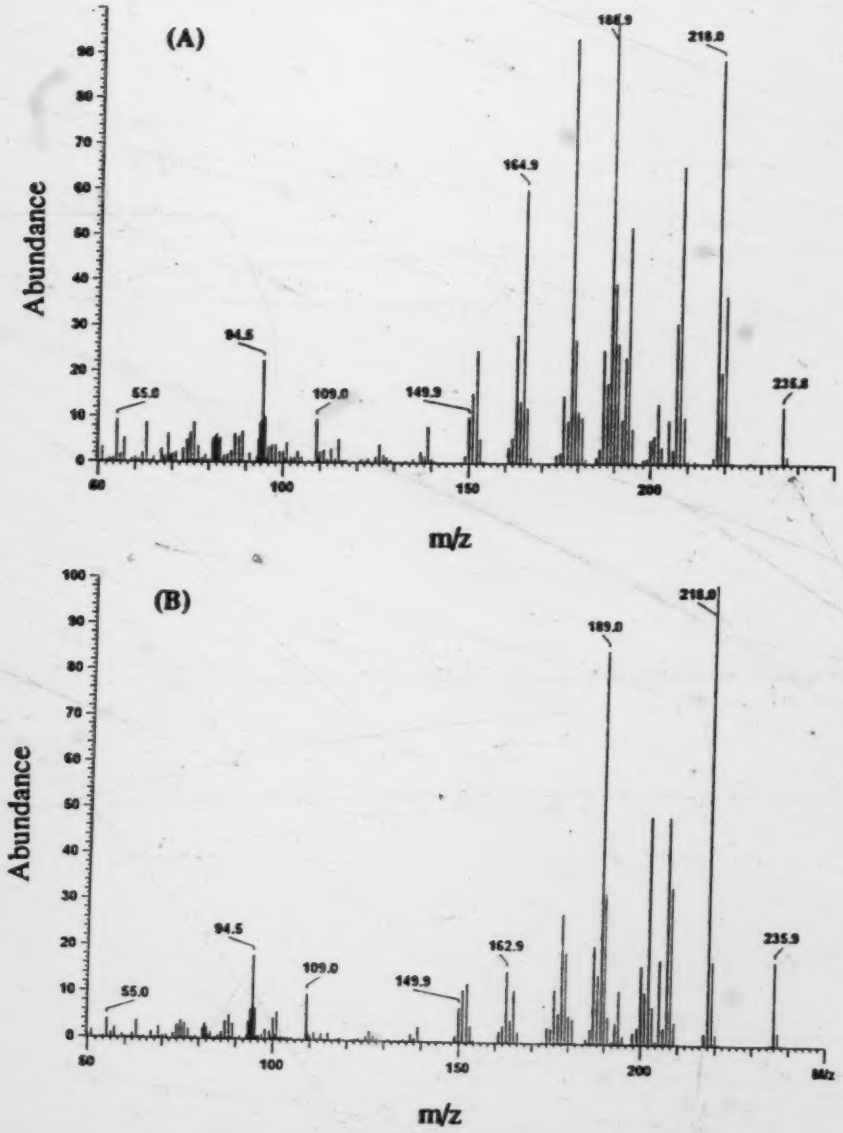


Fig. 59 Mass spectra of metabolite I (A) and II (B) found in the neutral extract of *Rhodococcus* S1 grown on anthracene in the presence of fluoranthene which were consistent with fluoranthene dihydrodiols.

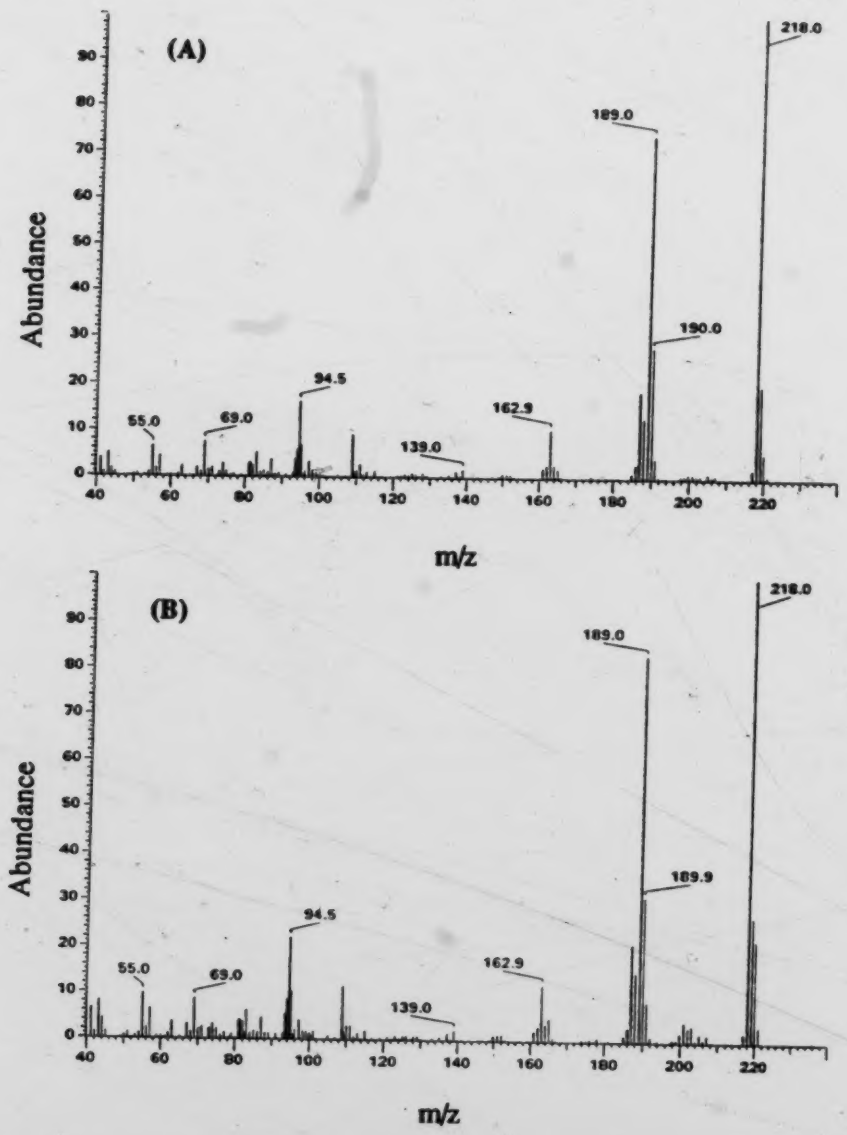


Fig. 60 Mass spectra of metabolite III (A) and IV (B) found in the neutral extract of *Rhodococcus* S1 grown on anthracene in the presence of fluoranthene which were consistent with hydroxyfluoranthene.

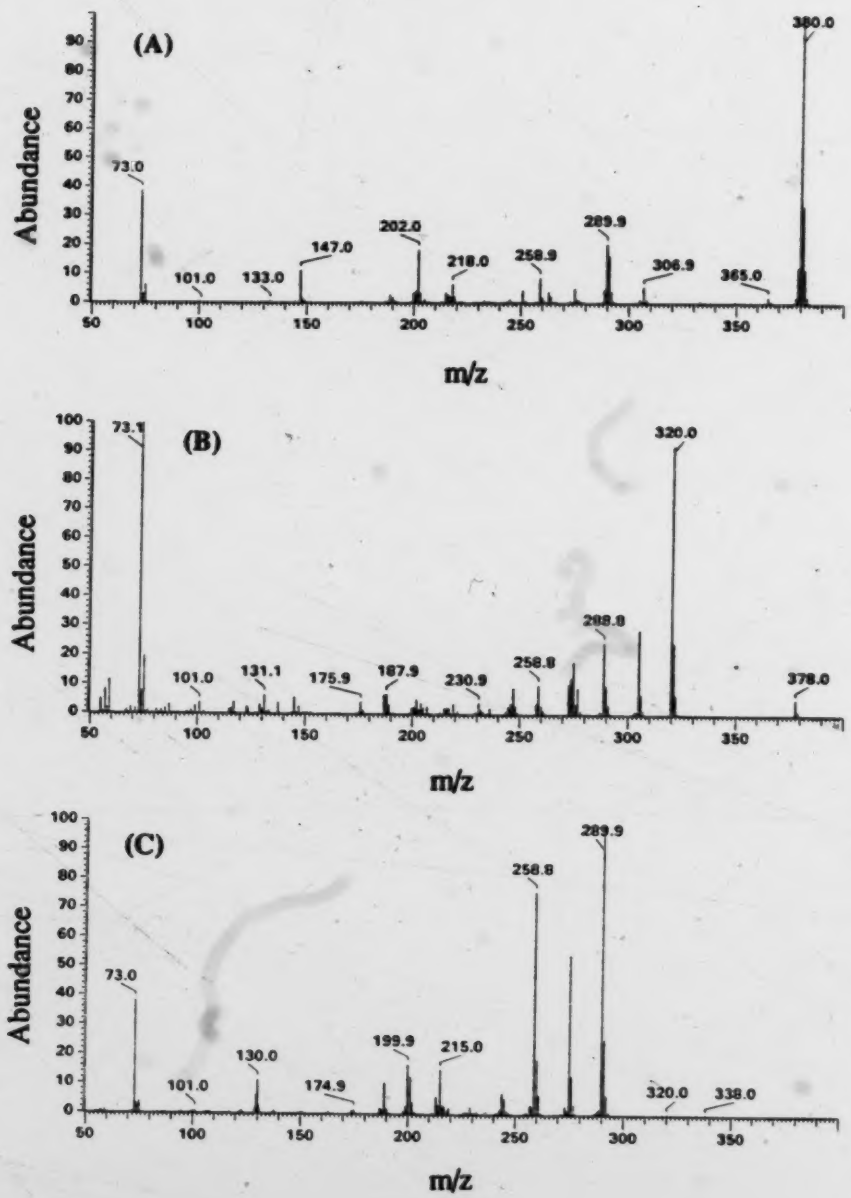


Fig. 61 Mass spectra of TMS derivatives of the tentatively identified fluoranthene dihydrodiol (A), dihydroxyfluoranthene (B), and fluoranthenol (C) produced from *Rhodococcus* S1 grown on anthracene in the presence of fluoranthene.

spectral analysis of that metabolite gave a M^+ at *m/z* 236 and major fragment ions at *m/z* 218 (base peak, M^+ -H_2O) indicating the loss of 18 mass unit which is normally observed in dihydrodiols (see explanation in section 4.3.1) (Fig. 62A). Derivatization with BSA gave a mass spectrum (Fig. 62B) that had a M^+ at *m/z* 380 indicating that two TMS groups had been incorporated into a molecule of the metabolite, consistent with the structure of a dihydrodiol of pyrene.

4.3.7 Cometabolism of chrysene.

GC analysis was performed on the neutral extract of a culture of *Rhodococcus* S1 grown on anthracene in the presence of chrysene. Mass spectral analysis of the largest metabolite peak, designated as compound I (Fig. 63A) revealed it had a M^+ at *m/z* 244 (base peak) and the next abundant ion fragment was at *m/z* 215 (M^+ -CHO). The molecular weight of 244 and fragmentation pattern, suggest that this compound I is likely an isomer of hydroxychrysene. However, after the neutral extract was derivatized with BSA, a di-TMS derivative of what is likely a chrysene dihydrodiol was found as the largest peak instead of a TMS derivative of hydroxychrysene. The TMS derivative of that metabolite produced a mass spectrum (Fig. 63B) that had a M^+ at *m/z* 406 indicating the incorporation of two groups of TMS which in turn implied that the underivatized compound possessed two hydroxyl groups. It is hypothesized that compound I was formed by dehydration of chrysene dihydrodiol. After derivatization, the TMS derivative of chrysene dihydrodiol in heated GC injection port liner did not undergo dehydration and was detected.

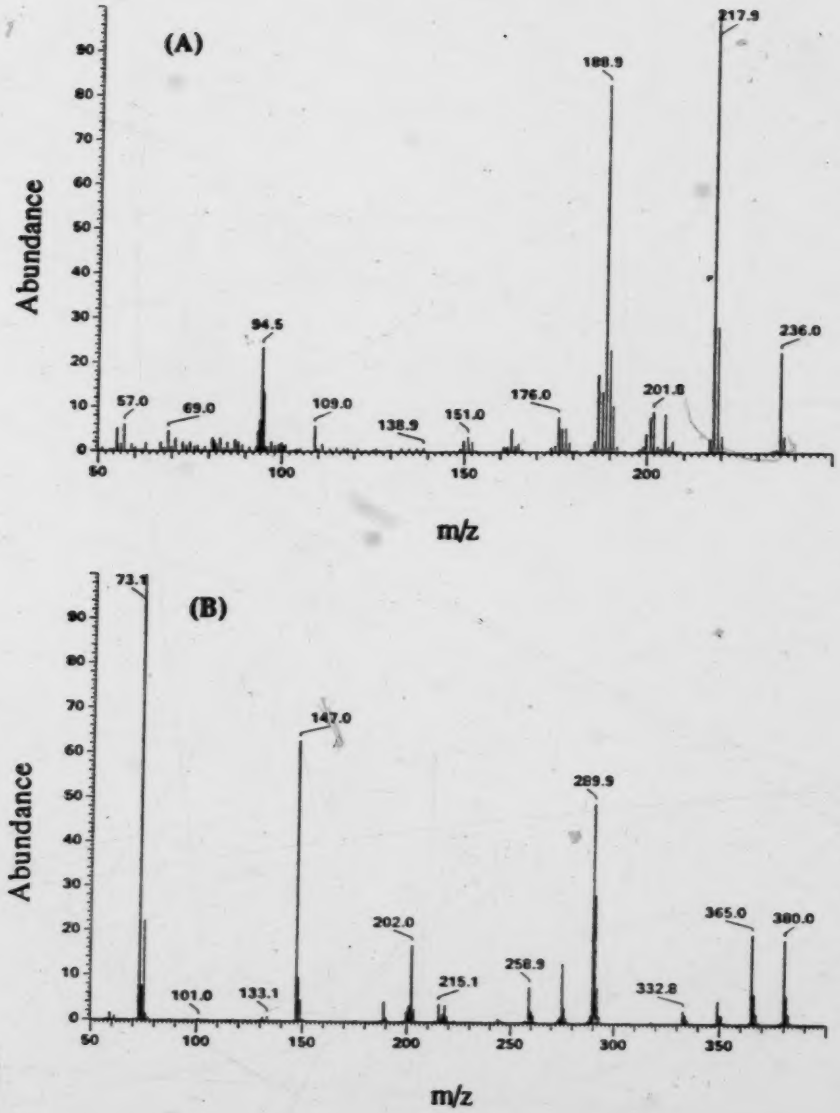


Fig. 62 Mass spectra of the metabolite (A) which corresponded to pyrene dihydrodiol and its TMS derivative (B) found in the neutral extract of *Rhodococcus* S1 grown on anthracene in the presence of pyrene.

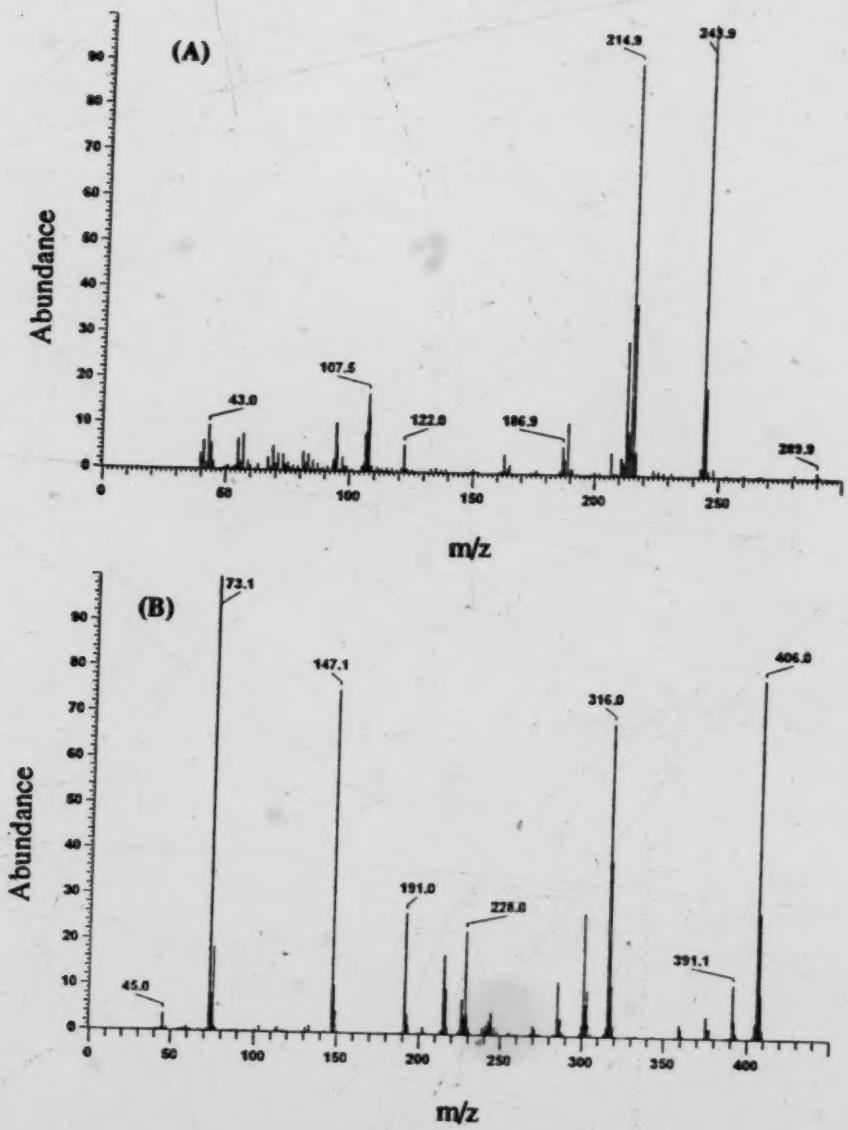


Fig. 63 Mass spectra of metabolite I found in the neutral extract of *Rhodococcus* S1 grown on anthracene in the presence of chrysene (A) and a TMS derivative found after the neutral extract was derivatized with BSA (B).

4.3.8 Cometabolism of 1,2-benzanthracene, naphthacene, and 2-methylphenanthrene.

It was found that *Rhodococcus* S1 did not transform 1,2-benzanthracene, naphthacene, or 2-methylphenanthrene to any of the predicted oxidation products. For example, in the case of 2-methylphenanthrene, no aromatic ring oxidation product was detected such as 2-methylphenanthrene dihydrodiol, nor was there any oxidation of the 2-methyl substituent to the corresponding phenanthryl alcohol, its aldehyde or acid. These data indicate that 1,2-benzanthracene, naphthacene, and 2-methylphenanthrene are not substrates for the enzyme system of *Rhodococcus* S1 causing cometabolism of the other PAHs described above.

4.4 Cometabolism of naphthalene, chrysene, and phenanthrene by *Rhodococcus* S1 grown on glucose as the sole carbon and energy source.

To investigate whether the enzyme responsible for cometabolism of PAHs by *Rhodococcus* S1 needed to be induced by growth on anthracene, PAH transformation was investigated using *Rhodococcus* S1 grown on glucose.

Washed cell pellets of *Rhodococcus* S1 grown on anthracene-MS were employed as inocula for growth on 1% glucose MS medium which contained either naphthalene or chrysene. GC analyses of the neutral extract of *Rhodococcus* S1 grown on glucose MS in the presence of naphthalene for 10 days (OD_{600} ~1) yielded the similar amount of naphthalene dihydrodiol as that of the bacterium when grown on anthracene MS in the presence of naphthalene. As expected, phenanthrene dihydrodiol was not found in this case because glucose was used as the growth substrate instead of anthracene. In a similar manner, chrysene dihydrodiol was found in the neutral extract of glucose MS plus chrysene culture and again phenanthrene dihydrodiol was not observed.

It was of interest to examine whether *Rhodococcus* S1 could retain the ability to cometabolize PAHs after being grown for several passages on glucose. Isolate S1 was grown on glucose MS medium in the presence of either naphthalene (1.95 mM) or phenanthrene (1.4 mM) for three consecutive batches. The bacterium grown on glucose without PAH was used as the control. In each flask when the bacterium had grown for three generations (based on the increase of OD_{600}), 10% of the culture was transfered to the next flask and the remaining 90% was extracted for dihydrodiol analysis. Naphthalene dihydrodiol was found only in the first passage. For the second and third passages, *Rhodococcus* S1 had a much longer lag phase of several days compared to the first passage and no naphthalene dihydrodiol was produced. The parallel experiments with phenanthrene gave different results. Similar yields of phenanthrene dihydrodiol were found in all three passages and only a minimal lag phase was observed. The data obtained from these experiments indicated that *Rhodococcus* S1 was able to retain its ability to cometabolize PAHs when it was grown on glucose as a sole source of carbon and energy and that naphthalene had an inhibitory effect on growth and transformation.

4.5 Cometabolism of phenanthrene and chrysene by anthracene-grown *Rhodococcus* S1 in the presence of cytochrome P450 inhibitors.

Cometabolism of phenanthrene and chrysene in the presence of cytochrome P450 monooxygenase inhibitors, metyrapone and ancymidol, was performed to examine if the transformation products of PAHs obtained from *Rhodococcus* S1 were oxidized by the action of cytochrome P450 monooxygenase. It was found that both compounds inhibited the formation of phenanthrene dihydrodiol and chrysene dihydrodiol in cultures of *Rhodococcus* S1 as summarized in Table 7.

These results suggested that *Rhodococcus* S1 cometabolically transformed PAHs by cytochrome P450-like monooxygenase activity and that the dihydrodiols were likely of the *trans* configuration.

Table 7 Effect of cytochrome P450 inhibitors on the formation of phenanthrene dihydrodiol and chrysene dihydrodiol by cultures of *Rhodococcus* S1.

		% Inhibition of formation	
Inhibitor	Concentration	Phenanthrene dihydrodiol	Chrysene dihydrodiol
Metyrapone	0.4 mM	33	27
	1.0 mM	100	82
Ancymidol	0.16 mM	14	38
	1.6 mM	100	100

5. DISCUSSION

5.1 *Rhodococcus* isolates and their growth on anthracene.

Three strains of acid-fast rhodococci were isolated from anthracene enrichment cultures of creosote-contaminated soil. Strain 11S and 2F1 were similar in their colonial morphology whereas strain S1 was clearly morphologically different from the other two strains. However, all three strains possessed similar biochemical, physiological, and chemical characteristics as described in Table 1.

In general, pure culture studies of the degradation of individual PAH have been directed at the ability of microorganisms to utilize PAH and the metabolic pathways involved. The information available for anthracene metabolism has been described mainly in Gram negative bacteria (Evans *et al.* 1965, Jerina *et al.* 1976, Menn *et al.* 1993, Sanseverino *et al.* 1993). To date, the only Gram positive bacteria reported to degrade anthracene are *Rhodococcus* sp. strain UW1 (Walter *et al.* 1991) and a coryneform bacillus strain SAntMu3 (Bouchez *et al.* 1996). However, metabolites were not identified in those studies and the degradative pathways used were not elucidated. These two bacterial strains required vitamin supplement in the mineral salts media for growth whereas *Rhodococcus* sp. strain 11S, S1, and 2F1 do not. *Rhodococcus* sp. strain UW1 was isolated by enrichment with pyrene (Walter *et al.* 1991). This strain was able to utilize not only pyrene but also anthracene, phenanthrene, fluoranthene, and chrysene as sole sources of carbon and energy. It also cometabolized naphthalene, dibenzofuran, fluorene, and dibenzothiophene with pyrene as the sole carbon and energy source. Bouchez *et al.* (1996) investigated the carbon distribution during PAH degradation in a coryneform bacillus strain SAntMu3. This strain was able to use anthracene as a sole source of carbon and energy but no information was presented to indicate if other PAHs were tested. *Rhodococcus* sp. strain S1 utilized only anthracene as the sole source of carbon and energy and

cometabolized naphthalene, biphenyl, acenaphthene, fluorene, phenanthrene, fluoranthene, pyrene, and chrysene. From the viewpoint of PAH biodegradation, it would appear that this strain does not produce enzymes which allow the cometabolites to enter the central metabolic pathways and support growth.

All three *Rhodococcus* isolates (strain 11S, S1, and 2F1) yielded similar growth curves when grown in complex media: TSB, BHI, and NB. They did not show a lag period in any of the three media. They grew better in TSB and BHI with higher growth rates and cell yield than in NB. In TSB and NB, after cell growth reached the stationary phase the cell populations gradually declined as normally seen in typical growth curves. For BHI, unlike the other two media, after the bacterial population reached stationary phase it declined sharply. This might not be an indication of cell death but of cell aggregation and settling.

During growth in anthracene-MS medium the three rhodococcal isolates displayed growth curves with unusual lag phases. As shown in Fig. 12 for strain S1, after inoculation cell populations (OD_{600}) dropped dramatically before entering the exponential growth phase, resulting in a two-day lag period. These growth experiments were performed in duplicate. All three isolates behaved similarly yielding growth curves with this consistent unusual lag phase whenever the organisms were grown in mineral salts medium with crystalline anthracene as the carbon source. The factor responsible for this type of lag phase might be the lipophilic type of cell wall containing mycolic acids that makes these bacteria tend to adhere to solid PAH substrates. It is believed that after inoculation, the bacteria begin to adhere to anthracene pellets which normally settle causing the observed optical density to drop sharply. After cells start growing, they are sloughed off the anthracene pellets, and the OD_{600} starts rising. The scanning electron micrograph in Fig. 14C showing *Rhodococcus* S1 growing on an anthracene pellet also supports this hypothesis. In addition, when the organisms were grown in mineral salts medium and

anthracene was supplied as a solution in *N,N'* -dimethylformamide, optical density was stable during the 1 day lag period before rising (Fig. 13) whereas the control growth curve with the anthracene supplied in large crystal form again displayed a lag phase with a sharp drop of OD_{600}.

When grown on mineral salts medium with crystalline anthracene *Rhodococcus* S1 yielded an OD_{600} of about 0.8. This low cell yield was not surprising. Low yields of *Pseudomonas* spp. were also reported when grown on aromatic hydrocarbons as the sole source of carbon and energy such as OD_{600} of 0.62 with 1-methylnaphthalene (Saftic and Fedorak 1993) and OD_{600} of slightly less than 1.0 with acenaphthene and acenapthylene (Komatsu *et al.* 1993).

Another interesting observation was that the mineral salts medium containing particulate anthracene always had fine anthracene particles floating on the surface of the liquid medium, due to its hydrophobic character. However, after the *Rhodococcus* isolates were inoculated and incubated the floating anthracene particles were more evenly suspended through the medium compared to the substrate control with no bacteria. This observation was also made in cometabolism experiments when the strain S1 was grown on anthracene salts medium in the presence of a variety of particulate PAHs. This finding implies that the *Rhodococcus* isolates may produce biosurfactants which reduce surface tension and enhance substrate solubilization.

Among the 11 PAHs tested as carbon sources, growth of *Rhodococcus* isolates was limited to only anthracene. While the three isolates could not grow on PAHs other than anthracene, these substrates were also examined in mineralization studies and the results confirmed the growth experiments in that only anthracene was mineralized as shown by the release of $^{14}CO_2$ from [9-^{14}C]anthracene but not from [9-^{14}C]phenanthrene or [U-^{14}C]naphthalene. One interesting aspect is the position of ^{14}C label that is on the middle ring in the 9-labelled compounds, which indicates that at least two of the three rings

of anthracene must be cleaved before any $^{14}CO_2$ can be released by the catabolic pathway for Gram negative bacteria. This confirms the conversion of anthracene to carbon dioxide and water that is an ultimate goal of employing microorganisms in the bioremediation of such toxic compounds in the environment. *Rhodococcus* sp. strain S1 mineralized about 85% of the added [9-^{14}C]anthracene within 3 days. No information on mineralization of anthracene by *Rhodococcus* sp. UW1 was given by Walter *et al.* (1991). For the coryneform bacillus strain SAntMu3,,Bouchez *et al.* (1996) determined the carbon balance of anthracene after 30 days of incubation by a carbon analyzer and reported that 65% of carbon was converted to carbon dioxide by this strain.

From growth experiments on substrate analogs, anthracenes substituted on C-2 by a methyl or chloride group were able to support growth of *Rhodococcus* sp. strain S1. As well, these substituents did not inhibit the bacterial growth on anthracene. However, when anthracene was substituted by a methyl group or chloride on C-1 or C-9, these compounds could not support growth of the isolate and prevented its growth on anthracene. These results may be a reflection of the intracellular anthracene dioxygenase specificity since there appears to be no barrier to entry for the anthracene analogs. These data imply that the dioxygenase from strain S1 may attack anthracene at the C-9a and C-1 or C-9 and C-9a positions. Even though the initial oxidation of anthracene by *Pseudomonas* spp. and *Beijerinckia* sp. has been reported to be at C-1 and C-2 positions to give anthracene *cis*-1,2-dihydrodiol (Evans *et al.* 1965, Jerina *et al.* 1976, Gibson and Subramanian 1984), it is not impossible for other positions to be oxidized first, for example between the bridgehead carbon and adjacent carbon. Recently angular dioxygenation has been proposed in the metabolic pathways for the initial ring oxidation of dibenzofuran by *Brevibacterium* sp. (Engesser *et al.* 1989), of carbazole by *Pseudomonas* sp. (Ouchiyama *et al.* 1993, Gieg *et al.* 1996), and the ring oxidation in the initial steps of dibenzofuran and fluorene by *Brevibacterium* sp., *Staphylococcus* sp., and *Pseudomonas* sp. (Strubel *et al.*

1991, Monna *et al.* 1993, Grifoll *et al.* 1994). Unlike the other bacteria reported to degrade anthracene, the *Rhodococcus* isolates described here are unusual in their very narrow PAH substrate specificity. The other bacterial strains are always able to utilize at least one or more other PAHs in addition to anthracene as sole sources of carbon and energy (Evans *et al.* 1965, Walter *et al.* 1991, Bouchez *et al.* 1995). There have been reports about other related PAH-metabolizing actinomycetes capable of utilizing more than one PAHs for growth. For example, *Mycobacterium* spp. are able to degrade fluorene, fluoranthene, phenanthrene, and pyrene as sole sources of carbon and energy (Boldrin *et al.* 1993, Tiehm and Frizsche 1995) or naphthalene, phenanthrene, fluoranthene, and others (Heitkamp *et al.* 1988, Kelley and Cerniglia 1991). Similarly, a *Corynebacterium* sp. isolated by growth on *o*-xylene was able to grow on other single-ring aromatics as well as naphthalene, tetrahydronaphthalene and other two-ring derivatives (Sikkema and De Bont 1993). The narrow PAH specificity of *Rhodococcus* sp. strain S1 suggests that metabolic pathway for the degradation of anthracene may yield some interesting information for comparison with the above strains.

The single feeding experiment designed to test intermediates of the *Pseudomonas* anthracene catabolic pathway as growth substrates for *Rhodococcus* gave little useful information as none of the compounds tested supported growth, including salicylate, a known inducer of the *nah* operon of *Pseudomonas* spp. Even though there was the possibility that the test compounds might be unable to enter the cells, it does not exclude the possibility that the *Rhodococcus* isolates have an anthracene metabolic pathway different from that proposed for *Pseudomonas* spp.

5.2 Growth of *Rhodococcus* S1 on crystalline anthracene.

There have been reports that *Mycobacterium* sp. can incorporate aliphatic hydrocarbons intact into their cell lipids by direct contact between the hydrophobic cell wall

and the hydrocarbon substrates (King and Perry 1975, Murphy and Perry 1983). In the growth experiments of *Rhodococcus* sp. strain S1 in anthracene MS medium, anthracene was added to the medium as a solid. Examination under phase-contrast and by scanning electron microsopy revealed that the bacterium colonized the surface of the anthracene crystals. This indicated that the hydrophobic cell wall of *Rhodococcus* sp. strain S1 allowed direct contact between the cells and the anthracene substrate which might subsequently facilitate the direct incorporation of the PAH into bacterial cells. To date, there have been no reports that crystalline PAHs are directly available for microbial degradation. Several researchers have studied substrate availability during naphthalene and phenanthrene biodegradation by *Pseudomonas* spp. and reported that the PAHs were utilized only in the soluble form (Wodzinski and Coyle 1974, Volkering *et al.* 1992 and 1993, Bouchez *et al.* 1995). Similarly, Tiehm and Fritzsche (1995) also reported that growth of *Mycobacterium* sp. was substrate-limited even in the presence of excess crystalline PAHs due to the low solubility and low dissolution rate of the compounds. However, before conclusions can be made about the growth of *Rhodococcus* S1 on crystalline anthracene, more investigation is needed to confirm whether the organism could utilize anthracene directly from the solid state.

5.3 Studies of metabolites from *Rhodococcus* spp. and *Pseudomonas* sp. strain HL4.

Preliminary chromatographic analyses of metabolites in the neutral and acid extracts of the supernatant from *Rhodococcus* spp. grown on anthracene-MS medium gave similar results for all three isolates. None of the metabolites matched any of the commercially available intermediates in the previously published anthracene catabolic pathway (see Fig. 3). However, few conclusions could be made from these negative results because they did not rule out the possibility that the organism might use a different pathway to that reported

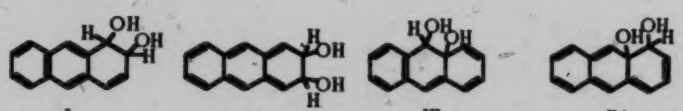
in pseudomonads or that the intermediates that matched commercially available standards were produced and metabolized further without accumulation in a detectable amount. For the comparative studies using *Pseudomonas* sp. strain HLA, the acid extract revealed 2-hydroxy-3-naphthoic acid as the major metabolite produced along with trace amounts of several unidentified metabolites. This confirmed that strain HLA uses a pathway similar to the one proposed for *Pseudomonas* spp. (Gibson and Subramanian 1984) to metabolize anthracene. Further metabolism of 2-hydroxy-3-naphthoic acid occurred at a slower rate than the initial oxidation of anthracene so that this metabolite accumulated in the culture supernatant. It has been reported that during the metabolism of naphthalene by several *Pseudomonas* spp., salicylate accumulates in high yield (Davies & Evans 1964, Shamsuzzaman & Barnsley 1974) since salicylate is metabolized at about 50% of the rate at which naphthalene is oxidized. Mahaffey *et al.* (1988) also suggested that the accumulation of *o*-hydroxypolyaromatic acids might be due to a rate-limiting reaction similar to that observed in naphthalene metabolism.

However, the neutral extract of *Rhodococcus* sp. strain S1, when grown on anthracene, always contained compound X as a major metabolite. Low and high resolution GC-MS analyses revealed that compound X had a molecular weight of 212, a chemical formula of $C_{14}H_{12}O_2$, and a mass spectral fragmentation pattern (Fig. 19A) that corresponded to anthracene dihydrodiol, the initial metabolite reported in the *Pseudomonas* anthracene catabolic pathway (Akhtar *et al.* 1975, Jerina *et al.* 1976). Since the authentic anthracene dihydrodiol was not commercially available, *Pseudomonas fluorescens* LP6a mutant D1, a Tn5 transposon mutant of the wild type strain LP6a (Foght and Westlake 1996) which is deficient in anthracene dihydrodiol dehydrogenase was used to produce anthracene dihydrodiol for comparative studies. Analysis by GC-MS of the neutral extract of strain LP6a-D1 and its TMS derivative also confirmed that the bacterium produced and accumulated anthracene 1,2-dihydrodiol. Interestingly when compound X was compared

with the strain LP6a-D1 anthracene dihydrodiol by TLC, GC-FID, and GC-MS analyses, it showed different Rf, retention time, and mass spectral fragmentation (see Fig. 19 & 20) on each test, respectively. These results clearly indicated that compound X was different from *Pseudomonas* anthracene dihydrodiol and might be a novel intermediate in anthracene metabolism by *Rhodococcus* sp. strain S1.

The degradative pathway of anthracene in Gram negative bacteria involves initial dioxygenase enzyme attack of anthracene at the C-1 and C-2 positions to give anthracene *cis*-1,2-dihydrodiol as the initial product (Fig. 3) (Cerniglia and Heitkamp 1989). To confirm indirectly if the anthracene dihydrodiol from strain LP6a-D1 had hydroxyl groups on the C-1 and C-2 positions, chemical dehydration of the neutral extract was performed. As expected, the resultant products were 1- and 2-anthrol confirmed by comparison with the chemically synthesized anthrol standards 1-, 2-, and 9-anthrol. The yield of 1- and 2-anthrol produced was 91% and 9%, respectively. This yield of 1-anthrol (91%) was similar to the yield of 1-anthrol (86-90%) reported by Jerina *et al.* (1976) for dehydration of anthracene dihydrodiol from *Pseudomonas* sp.

When grown on anthracene *Rhodococcus* S1 always produced compound X with a chemical formula $C_{14}H_{12}O_2$, a molecular weight of 212, and two hydroxyl groups. Considering the symmetrical structure of anthracene, there are only four possible locations for attack by anthracene dioxygenase enzyme that would yield a metabolite with the chemical properties mentioned above, i.e., between carbons 1&2, 2&3, 9 & 9a, and 9a and 1 as shown in the figures below.



I II III IV

Structures III and IV would require angular dioxygenation to occur. Angular dioxygenation reactions have been reported for metabolism of fluorene, carbazole, and dibenzofuran (Engesser *et al.* 1989, Ouchiyama *et al.* 1993, Grifoll *et al.* 1994). Thus, the activity of *Rhodococcus* sp. strain S1 to grow on 2-methyl anthracene but not 1- or 9-methyl anthracene was compatible with structure III and IV. Since the bacterium produces compound X only in a very small amounts, attempting to purify it for chemical structure identification by NMR analysis was not successful. One indirect method to prove its structure was to use tests similar to those performed for identification of *P. fluorescens* LP6a mutant D1 anthracene dihydrodiol, that is, to perform chemical dehydration to obtain phenolic product(s) which could be compared with authentic standards. Thus after dehydration, structures I, II, III, and IV would be expected to yield a mixture of 1- and 2-anthrol, 2-anthrol alone, 9-anthrol alone, and 1-anthrol alone, respectively. So compound X was chemically dehydrated to give a single compound Y which had a molecular weight 194, a chemical formula $C_{14}H_{10}O$, and one hydroxyl functional group as indicated by low and high resolution GC-MS and GC-FTIR. Compound Y appeared to be consistent with the structure of anthrol. Surprisingly, however, the GC retention time of compound Y did not match any of the 1-, 2-, or 9-anthrol standards, which in turn implied that compound X could not be any of the four structures shown above. The conclusion was made at this point was that while strain LP6a-D1 did produce anthracene 1,2-dihydrodiol as the initial metabolite as reported in the proposed anthracene catabolic pathway, the neutral metabolite found from *Rhodococcus* S1 grown on anthracene could not be anthracene dihydrodiol even though the molecular weight and chemical formula were consistent with a dihydrodiol of a three-ring PAH. The only possibility left was that this metabolite might come from phenanthrene, an isomer of anthracene, which is present as a <1% contaminant in commercial 'pure' anthracene. Therefore, identification of the structure of compound X as

phenanthrene dihydrodiol was pursued by comparing its dehydration product(s) with standard phenanthrols.

There are reports that initial sites of enzymic attack of phenanthrene by bacteria may vary. *Pseudomonas* and *Beijerinckia* oxidize phenanthrene at carbons 1,2 and 3,4-positions to form phenanthrene *cis*-1,2-dihydrodiol and phenanthrene *cis*-3,4-dihydrodiol (Cerniglia and Heitkamp 1989). Attack at the 9,10 position to form phenanthrene *trans*-9,10-dihydrodiol by *Streptomyces flavovirens* has been reported (Sutherland *et al.* 1990). These three isomers of dihydroxy dihydrophenanthrene would yield 1- and 2-phenanthrol, 3- and 4-phenanthrol, and 9-phenanthrol, respectively, upon dehydration. Interestingly, compound Y matched 9-phenanthrol which implied that compound X must be phenanthrene 9,10-dihydrodiol. These data indicated that *Rhodococcus* sp. strain S1 cometabolized phenanthrene at the C-9 and C-10 positions to yield phenanthrene 9,10-dihydrodiol which gave 9-phenanthrol on chemical dehydration. This result was confirmed by the increased amount of compound Y which accumulated when phenanthrene was added to the anthracene-MS medium used for growth of *Rhodococcus* S1 (see Fig. 37). In addition, the GC-MS fragmentation patterns of compound X and its TMS and BTB derivatives were compared with phenanthrene 1,2- and 3,4-dihydrodiol produced by strain LP6a-D1 and their TMS and BTB derivatives. These results confirmed that compound X was neither of these two biologically produced standards.

Phenanthrene *trans*-9,10-dihydrodiol has been reported as the metabolite produced from phenanthrene by fungi (Cerniglia and Yang 1984, Cerniglia *et al.* 1989, Sutherland *et al.* 1993, Bezalel *et al.* 1996a), a cyanobacterium (Narro *et al.* 1992), and an actinomycete (Sutherland *et al.* 1990). These microorganisms did not utilize phenanthrene as the sole source of carbon and energy for growth but cometabolized it by the action of a cytochrome P450 monooxygenase enzyme to form phenanthrene *trans*-9,10-dihydrodiol. Similarly, *Rhodococcus* S1 cannot utilize phenanthrene as a sole source of carbon and energy, but

when grown on anthracene it can cometabolize the small amounts of phenanthrene (less than 5 mg/l medium) to phenanthrene 9,10-dihydrodiol. Hence it is likely that this S1 isolate utilizes a cytochrome P450-like monooxygenase enzyme to oxidize phenanthrene to the accumulated phenanthrene *trans*-9,10-dihydrodiol. However, surprisingly the BTB derivative of compound X clearly showed that only one rather than two molecules of BTB had reacted with the two hydroxyl groups on the compound. It was assumed that the PAH diols in the *trans* -configuration would require two molecules of BTB to form the boronic acid derivatives, since Sugihara and Bowman (1958) have shown that *cis*-1,2-diols of cyclohexane and cyclopentane were derivatized by phenylboronic acid in a 1:1 ratio whereas trans isomers behaved in a 1:2 ratio. Brooks and Maclean (1971) reported that *cis*- and *trans*-2-hydroxycyclohexanecarboxylic acid were derivatized with n-butylboronic acid in a similar manner by incorporating only one molecule of BTB. There have been some other papers reporting the utilization of various boronic acid derivatives to derivatize diol compounds, i.e., methoxyethylphenylboronic acid with bacterial and synthetic *cis*-diols (Resnick *et al.* 1995), phenylboronic acid with anisole and phenetole *cis*-dihydrodiol (Resnick and Gibson 1993), benzeneboronic acid and ferroceneboronic acid with various kinds of diol compounds (Gamoh *et al.* 1994), and 1-butaneboronic acid with naphthalene *cis*-1,2-dihydrodiol (Wilson and Madsen 1995). To date there has been no report on the boronate ethers of polycyclic aromatic *trans*-diols, and it is still inconclusive whether the derivatization of phenanthrene *trans*-9,10-dihydrodiol would require one or two molecules of boronic acid to form the derivative. However, GC-MS analysis also revealed that the phenanthrene dihydrodiol formed by *Phanerochaete chrysosporium* grown on Kirk's medium in the presence of phenanthrene required only one molecule of BTB for derivatization (data not shown). In addition, phenylboronic acid was also employed to derivatize phenanthrene 9,10-dihydrodiol from *Rhodococcus* S1. Again the resultant phenylboronate ether analyzed by GC-MS revealed that only one molecule of BTB

incorporated into the compound (data not shown). The inconclusive data on stereochemistry of phenanthrene 9,10-dihydrodiol from *Rhodococcus* sp. strain S1 prompted the preparation of phenanthrene *trans*-9,10-dihydrodiol by chemical synthesis to be used as a standard compound. It was not surprising that the GC retention times and mass spectral fragmentation pattern of phenanthrene *trans*-9,10-dihydrodiol and its TMS and BTB derivatives from *Rhodococcus* sp. strain S1 (see Fig. 19 and 36) were identical to those of standard phenanthrene *trans*-9,10-dihydrodiol (see Fig. 41 and 42).

The data obtained to this point suggested that the chemical structure of compound X would likely be phenanthrene *trans*-9,10-dihydrodiol. Thus, isolate S1 likely oxidized phenanthrene using a cytochrome P450-like monooxygenase. This proposal was supported by data obtained from the cometabolism of phenanthrene and chrysene in the presence of two cytochrome P450 inhibitors. Addition of metyrapone and ancymidol, the cytochrome P450 monooxgenase inhibitors (Oikawa *et al.* 1988 &1992), decreased the amount of the transformation product phenanthrene *trans*-9,10-dihydrodiol produced by strain S1(see Table 7). These results were similar to those reported for the white rot fungus *Pleurotus ostreatus* (Bezalel *et al.* 1996) in which less phenanthrene *trans*-9,10-dihydrodiol was observed when cytochrome P450 inhibitors, 1-aminobenzotriazole, proadifen, and fluoxetine were present in the medium. Since *Rhodococcus* S1 has been shown to cometabolize some other PAHs, the effect of cytochrome P450 inhibitors on the formation of chrysene dihydrodiol was also examined to confirm the phenanthrene oxidation observations. Chrysene dihydrodiol was chosen in the study because it lends itself well in to our analytical methods. Using GC analysis, the TMS derivative of chrysene dihydrodiol has a long GC retention time (40.17 min) which gave an isolated peak without interference by peaks of other unidentified metabolites, or the inhibitors metyrapone and ancymidol. Similar results were obtained with both metyrapone and ancymidol inhibiting the formation of chrysene dihydrodiol. These data support the

conclusion that *Rhodococcus* S1 uses a cytochrome P450-like monooxygenase to transform non-growth substrate PAHs to accumulated oxidized products.

5.4 Evidence supporting the involvement of cytochrome P450-like monooxygenase in the cometabolic transformation of non-growth substrate PAHs by *Rhodococcus* S1

Rhodococcus S1 was unable to use PAHs other than anthracene as the sole source of carbon and energy for growth. However, the isolate was able to cometabolize various PAHs to oxidized products which accumulated when grown on anthracene. Evidence supporting the involvement of a cytochrome P450-like monooxygenase in the cometabolic transformation of non-substrate PAHs by *Rhodococcus* S1 is as follows:

1. *Rhodococcus* S1 cometabolically transformed phenanthrene to phenanthrene *trans*-9,10-dihydrodiol. Coinjection of phenanthrene 9,10-dihydrodiol and its TMS derivative from *Rhodococcus* S1 with the chemically synthesized phenanthrene *trans*-9,10-dihydrodiol and its TMS derivative resulted in coelution of those compounds. BTB derivatives of those two compounds also had similar GC retention times.

In addition, the mass spectra of phenanthrene 9,10-dihydrodiol and its TMS and BTB derivatives from *Rhodococcus* S1 had the same fragmentation pattern as those of chemically synthesized phenanthrene *trans*-9,10-dihydrodiol (see Fig. 19, 36, 41, and 42).

2. Cometabolic transformation of phenanthrene and chrysene by *Rhodococcus* S1 was inhibited by cytochrome P450 inhibitors (see Table 7).

3. Dihydroxynaphthalene obtained as the cometabolized product of naphthalene was not the 1,2- or 2,3-isomer since the compound and its TMS ether had different GC retention times from those of authentic 1,2- and 2,3-dihydroxynaphthalene and their TMS ethers. Dihydroxynaphthalene derived by the action of a dioxygenase enzyme would have the two hydroxy groups on adjacent carbon atoms (see Fig. 8). Thus,

dihydroxynaphthalene from *Rhodococcus* S1 would likely be derived by the action of a monooxygenase enzyme repeatedly attacking naphthalene.

This evidence suggests that phenanthrene *trans*-9,10-dihydrodiol and chrysene dihydrodiol were likely to be the products of a cytochrome P450-like monooxygenase from *Rhodococcus* S1. The transformation products of other non-growth substrate PAHs derived from cometabolism by strain S1 were also likely products of cytochrome P450-like monooxygenase activity and the dihydrodiols of naphthalene, fluoranthene, pyrene, and chrysene detected were likely in the *trans* -configuration.

5.5. Cometabolism of various PAHs by *Rhodococcus* S1 when grown on anthracene.

Since *Rhodococcus* sp. strain S1 appeared to demonstrate cytochrome P450-like monooxygenase activity when grown on anthracene to produce phenanthrene dihydrodiol and chrysene dihydrodiol it was decided to study the substrate specificity of the monooxygenase using a variety of PAHs.

5.5.1 Cometabolism of naphthalene.

During growth of *Rhodococcus* S1 on anthracene in the presence of naphthalene, at least five metabolites were produced that were potentially oxidation products of naphthalene. Due to the limitation of authentic standards for comparison, only two metabolites were positively identified. They were 1-naphthol and 1,4-naphthoquinone. The other three metabolites were tentatively identified as naphthalene dihydrodiol, dihydroxynaphthalene, and naphthoquinone.

Since evidence suggested that *Rhodococcus* S1 might utilize cytochrome P450-like monooxygenase to oxidize phenanthrene to a *trans* dihydrodiol as discussed earlier, it was more likely that naphthalene dihydrodiol produced by strain S1 would be in the *trans*

configuration but the specific structure was not obtained. Metabolite III was identified as dihydroxynaphthalene. This compound was proved to be not a 1,2- or 2,3-dihydroxynaphthalene but the location of hydroxy groups was not known. The extensive reviews on fungal metabolism of PAHs by Cerniglia *et al.* (1992) reported several principal metabolites produced from naphthalene by several strains of fungi via the action of cytochrome P450 monooxygenase including naphthalene *trans*-1,2-dihydrodiol, 1-naphthol, 2-naphthol, 1,2-naphthoquinone, 1,4-naphthoquinone, 4-hydroxy-1-tetralone, and sulfate and glucuronide conjugates. Narro *et al.* (1992) also reported that a marine cyanobacterium *Oscillatoria* sp. oxidized naphthalene predominantly to 1-naphthol via naphthalene 1,2-oxide which indicated the action of cytochrome P450 monooxygenase. However, there have been recent papers reporting bacterial monohydroxylation of benzocyclic compounds, at the benzylic aromatic portion. Eaton *et al.* (1996) reported the monooxygenation of 1,2-dihydronaphthalene to form 2-hydroxy-1,2-dihydronaphthalene by the monooxygenase of *Sphingomonas* sp. Monohydroxylation of various aromatic compounds catalyzed by naphthalene dioxygenase has also been reviewed by Resnick *et al.* (1996). Therefore, we cannot exclude the repeatedly monohydroxylation as the mechanism employed by strain S1 to yield dihydroxynaphthalene.

Although there have been reports of quinone formation from PAHs by fungi (Vyas *et al.* 1994, Bezalel *et al.* 1996) and a cyanobacterium *Agmennellum* sp. (Narro *et al.* 1992), there have no such reports for other bacteria. However, in this study two isomers of napthoquinone were found as the oxidation products of *Rhodococcus* S1.

5.5.2 Cometabolism of biphenyl.

A review by Safe (1984) of the bacterial metabolism of biphenyls, which focused primarily on Gram negative strains, reported that bacteria initially oxidize biphenyl by a dioxygenase enzyme at the C-2 and C-3 positions to yield biphenyl *cis*-2,3-dihydrodiol

which is then oxidized to 2,3-dihydroxybiphenyl prior to cleavage of the aromatic nucleus. Biphenyl *cis*-2,3-dihydrodiol has also been reported by Resnick *et al.* (1996) as a product of the dioxygenation reaction of *Pseudomonas* naphthalene dioxygenase enzyme with biphenyl. However, *Rhodococcus* S1 did not utilize biphenyl for growth, but cometabolized it to the accumulated products: 2- and 4-hydroxybiphenyl and a dihydroxybiphenyl. Since *Rhodococcus* S1 is more likely to metabolize biphenyl via a cytochrome P450-like monooxygenase enzyme, the products are more consistent with those reviwed on fungal metabolism of biphenyl by Gibson and Subramanian (1984). In contrast to bacteria, the major site of fungal hydroxylation is usually at the 4-position, with significantly less oxidation occuring at C-2 and C-3. Several oxidation products of biphenyl by fungi have been reported: 4-hydroxybiphenyl as the major metabolite, 2-hydroxybiphenyl, 3-hydroxybiphenyl, 4,4'-dihydroxybiphenyl, and 2,5-dihydroxybiphenyl. Gibson and Subramanian (1984) suggested that fungi could oxidize biphenyl to phenols by direct hydroxylation or isomerization of arene oxide intermediates.

5.5.3 Cometabolism of acenaphthene.

A *Beijerinckia* sp. which could not utilize acenaphthene as the sole source of carbon and energy for growth has been reported to cometabolize acenaphthene (Schoken and Gibson 1984). When grown on succinate in the presence of acenaphthene the bacterium oxidized the five-membered ring by monooxygenation to form metabolites that included 1-acenaphthenol, 1-acenaphthenone, acenaphthene *cis*-1,2-dihydrodiol, acenaphthenequinone, and possibly 1,2-dihydroxyacenaphthylene. Komatsu *et al.* (1993) isolated a strain of *Pseudomonas* sp. on the basis of its ability to utilize acenaphthene as the sole source of carbon and energy for growth. They reported that acenaphthene was metabolized via 1-acenaphthenol and 1-acenaphthenone. Selifonov *et al.* (1996) reported the action of naphthalene dioxygenase expressed from the cloned genes of plasmid NAH7

from *Pseudomonas* on acenaphthene. They described that acenaphthene underwent monooxygenation to 1-acenaphthenol with subsequent conversion to 1-acenaphthenone and *cis*- and *trans*-acenaphthene-1,2-diols. *Rhodococcus* S1 does not utilize acenaphthene as carbon and energy source for growth. However, when this isolate was grown on anthracene in the presence of acenaphthene it produced acenaphthenol and acenaphthenone as identified by GC-MS. The mass spectra of acenaphthenol and acenaphthenone from *Rhodococcus* S1 were similar to those derived from *Beijerinckia* sp. (Schoken and Gibson 1984) and *Pseudomonas* sp. (Selifonov *et al.* 1996) for acenaphthenol (*m/z* 170 $[M^+]$, 169 $[M-H]^+$, 152 $[M-H_2O]^+$) and acenaphthenone (*m/z* 168 $[M^+]$, 140 $[M-CO]^+$), respectively.

5.5.4 Cometabolism of fluorene.

During growth of *Rhodococcus* S1 on anthracene in the presence of fluorene, two neutral metabolic products were detected. Based on mass spectrometry, they were both identified as fluorenol. Only one minor metabolite could be positively identified as 2-fluorenol by comparison with an authentic 2-fluorenol based on a match of mass spectra and GC retention time. The major fluorenol was found not to be 9-fluorenol but the specific isomer could not be confirmed due to unavailability of fluorenol authentic standards.

Casellas *et al.* (1997) reported their recent findings that *Arthrobacter* sp. strain F101 utilized 3 independent pathways to degrade fluorene. Two productive routes were initiated by dioxygenation at positions 1,2 and 3,4 respectively which eventually allow the utilization of all fluorene carbons for growth. The alternative, non-productive, pathway was initiated by monooxygenation at C-9 to give 9-fluorenol and then 9-fluorenone. A *Mycobacterium* sp. also degraded fluorene by cometabolism and accumulated 9-fluorenol and 9-fluorenone as dead-end products (Boldrin *et al.* 1993). *Staphylococcus auriculans*

also hydroxylated C-9 of fluorene by a monooxygenase to form 9-fluorenol and 9-fluorenone (Monna *et al.* 1993). Similarly to bacteria, the fungus *Cunninghamella elegans* oxidized fluorene at the C-9 position to form 9-fluorenol and 9-fluorenone (Pothuluri *et al.* 1993) as did many other filamentous fungi.

Unlike the other reports of fluorene degradation, *Rhodococcus* S1, did not oxidize fluorene at the C-9 position as shown by identification of the accumulated fluorenols. The minor fluorenol accumulated by strain S1 was 2-fluorenol. Since 2-fluorenol has not been reported before as a metabolite in the oxidation of fluorene and the major fluorenol accumulated by strain S1 is not 9-fluorenol which has been reported in several papers (Grifoll *et al.* 1992, Pothuluri *et al.* 1993, Grifoll *et al.* 1994 and 1995, Casellas *et al.* 1997), the existence of a metabolic pathway for the degradation of fluorene by *Rhodococcus* sp. strain S1 has yet to be elucidated.

5.5.5 Cometabolism of fluoranthene.

When grown on anthracene in the presence of fluoranthene, *Rhodococcus* S1 produced some metabolites tentatively identified by GC-MS as fluoranthene dihydrodiols, hydroxyfluoranthenes, and dihydroxyfluoranthene. There have been reports of bacteria capable of transforming fluoranthene by cometabolism but few details have been reported on its metabolites (Barnsley 1975) or utilizing it for growth (Mueller *et al.* 1990, Weissenfels *et al.* 1990 and 1991, Walter *et al.* 1991). The metabolic intermediates of fluoranthene reviewed by Sutherland *et al.* (1995) including 7-acenaphthenone, 7-hydroxyacenaphthylene, 3-hydroxymethyl-4,5-benzocoumarin, 9-fluornone-1-carboxylic acid, 8-hydroxy-7-methoxyfluoranthene, 9-hydroxyfluorene, 9-fluorenone, 1-acenaphthenone, 9-hydroxy-1-fluorenecarboxylic acid, phthalic acid, 2-carboxybenzaldehyde, benzoic acid, phenylacetic acid, and adipic acid. However, in these studies *Rhodococcus* S1 seemed to transform fluoranthene without any evidence of ring

cleavage whereas most of the fluoranthene metabolites produced by bacteria reported by other workers had at least one ring of fluoranthene broken.

5.5.6 Cometabolism of chrysene.

Chrysene dihydrodiol was detected as the product of chrysene cometabolism by *Rhodococcus* S1. Chrysene has been reported to be utilized as a sole carbon and energy source for growth of a *Rhodococcus* sp. (Walter *et al.* 1991) and a *Pseudomonas* sp. (Caldini *et al.* 1995), but no details of its degradative pathway or metabolites were reported in those studies. However, there have been reports of some oxidation products of chrysene from filamentous fungi. For example, Pothuluri *et al.* (1994) reported 2-hydroxychrysene and sulfate conjugates of 2,8-dihydroxychrysene as the major metabolites from *Cunninghamella elegans* whereas Kiehlmann *et al.* (1996) reported chrysene *trans*-1,2-dihydrodiol as the transformation product of chrysene from a few isolates of *Penicilliun* and *Syncephalastrum*. To my knowledge, chrysene dihydrodiol produced by *Rhodococcus* S1 is the first chrysene metabolite reported from bacteria.

5.5.7 Cometabolism of 2-methylphenanthrene.

In this study, *Rhodococcus* S1 was able to utilize anthracene and 2-methyl anthracene for growth and to cometabolize phenanthrene but not 2-methyl phenanthrene. The anticipated product of aromatic ring oxidation, 2-methyl phenanthrene dihydrodiol, was not detected, nor were any methyl substituent oxidation products detected such as 2-methyl phenanthryl alcohol, its aldehyde or acid.

Oxidation of methyl substituted aromatic compounds by various strains of bacteria has been previously reported. Cerniglia (1984), reported that a single methyl group on a single-ring aromatic compound, toluene, can either be initially oxidized at the methyl group to give benzoic acid via benzyl alcohol and benzyl aldehyde or on the

aromatic ring to form a dihydrodiol, depending on the organisms involved. However, there are reports that a methyl group on an aromatic compound containing two benzene rings is not susceptible to oxidation by heterotrophic bacteria. For example, the methyl group remained intact during the initial metabolism of 1-methyl naphthalene (Dean-Raymond and Bartha 1975), 2-methyl naphthalene (Williams *et al.* 1975), and 3- and 4-methylbiphenyl (Fedorak and Westlake 1983). In contrast, Kropp *et al.* (1994 and 1996) reported that the 1-methyl naphthalene degrading *Pseudomonas* oxidized 1-methyl naphthalene to 1-methyl naphthol and naphthoic acid. Selifonov *et al.* (1996) reported the action of naphthalene dioxygenase enzyme expressed from the cloned genes of plasmid NAH7 on a variety of methyl-substituted aromatic compounds. This enzyme catalyzed monooxygenation reaction of the methyl group to form benzylic alcohols which were subsequently converted to the corresponding carboxylic acids. They made a case that monooxygenation of benzylic methyl groups was strongly predominant over aromatic ring dioxygenation. However, in the methyl-substituted aromatic sulfur heterocycle 3-methylbenzothiophene, two different monooxygenase reactions predominated. These were: (i) oxidation of the methyl group to form 3-hydroxymethylbenzothiophene and benzothiophene-3-carboxylic acid (ii) sulfoxidation without oxidation of the methyl group to give 3-methylbenzothiophene sulfoxide.

5.5.8 Summary of cometabolism.

Normally bacterial growth on PAHs requires a dioxygenase enzyme to oxidize PAHs to the respective dihydrodiols in the *cis*-configuration for further metabolism while monooxygenase enzymes, mainly from fungi, oxidize PAHs to their respective *trans*-dihydrodiols which are not metabolized further, resulting in dead-end products.

In this study, *Rhodococcus* S1 was shown by GC-MS analyses to cometabolize 8 out of 11 PAHs tested when the bacterium was grown on anthracene and at least one

oxidation product from each PAH cometabolized was detected by the gas chromatographic methods used. These products are summarized in Table 8 below.

Table 8 Cometabolism of various PAHs by *Rhodococcus* S1 grown on anthracene.

PAH	Products
Naphthalene	Naphthalene dihydrodiol[b], Dihydroxynaphthalene[b], 1-Naphthol[a], 1,4-Naphthoquinone[a], Naphthoquinone[b]
Biphenyl	Dihydroxybiphenyl[b], 2-Hydroxybyphenyl[a], 4-Hydroxybiphenyl[a]
Acenaphthene	Acenaphthenol[b], Acenaphthenone[b]
Phenanthrene	Phenanthrene *trans*-9,10-dihydrodiol[a]
Fluorene	2-Fluorenol[a], Fluorenol[b], Dihydroxyfluorene[b]
Fluoranthene	Fluoranthene dihydrodiol[b] (2 isomers), Dihydroxyfluoranthene[b] Hydroxyfluoranthene[b] (2 isomers),
Pyrene	Pyrene dihydrodiol[b]
Chrysene	Chrysene dihydrodiol[b]
1,2-Benzanthracene	No oxidation products detected
Naphthacene	No oxidation products detected
2-methyl phenanthrene	No oxidation products detected

[a] Identification was based on match of mass spectral fragmentation and GC retention times with those of authentic samples.

[b] Identification was based on molecular ion and mass spectral fragmentation.

It has been shown earlier that this isolate utilized a cytochrome P450-like monooxygenase to metabolize phenanthrene and chrysene to phenanthrene *trans*-9,10-dihydrodiol and chrysene dihydrodiol respectively. While cytochromes P450 may be highly specific or have relatively broad specificity (Munro and Lindsay 1996), this cytochrome P450-like monooxygenase of *Rhodococcus* S1 seemed to have relaxed substrate specificity since it could cometabolize several kinds of PAH. It has frequently been shown among microorganisms that an individual strain can oxidize more than one kind of PAHs (see Table 4) for growth. In contrast, it is presumed that *Rhodococcus* S1 possesses a dioxygenase enzyme that has a narrow substrate specificity since among 12 PAHs tested it can utilize only anthracene as a growth substrate. There have been extensive studies on the naphthalene dioxygenase enzyme from *Pseudomonas* spp. describing the relaxed substrate specificity of this enzyme which catalyzes dioxygenation of naphthalene and many related 2- and 3-ring aromatic and naphthenoaromatic compounds to their corresponding *cis*-diols (Resnick *et al.* 1996, Gibson *et al.* 1995, Selifonov *et al.* 1996). In addition to these dioxygenase reactions, this naphthalene dioxygenase enzyme also has desaturase, monooxygenase, and sulfoxygenase activities. It is still unclear whether *Rhodococcus* S1 produces a single enzyme that has both monooxygenase and dioxygenase activities or produces at least two types of enzymes i.e. monooxygenase and dioxygenase.

5.6 Unidentified metabolites.

In addition to the data presented earlier there are some additional results (data not shown) which might be useful for future studies. For example, there is a trace amount of another metabolite found during GC analysis of the extracts of spent medium of *Rhodococcus* S1 when it is grown on anthracene which shows a molecular ion at *m/z* 212 and a mass spectrum corresponding to dihydrodiol of a three ring aromatic hydrocarbon.

This compound could be a metabolite of either anthracene or phenanthrene because phenanthrene was always present in the commercial anthracene used for *Rhodococcus* S1 growth. The compound was present in a tiny amount so its identification was not pursued. There was also evidence of a trace amount of 9-anthrol present in the spent medium of *Rhodococcus* S1 based on a match of mass spectral fragmentation and GC retention time with the standard 9-anthrone and the TMS ether of 9-anthrol. This compound was always present as 9-anthrone (keto form) but reacts as if it had a hydroxyl group when derivatized with BSA to give a TMS ether of 9-anthrol. A small amount of a compound consistent with a dihydroxy-substituted three ring PAH was always detected when the neutral extract of *Rhodococcus* S1 was analyzed by GC. It can be either dihydroxyanthracene or dihydroxyphenanthrene. This study is of necessity incomplete and further experimentation is needed to clarify these points.

6. APPLICATIONS.

6.1. Application for use as a member of a mixed culture.

An ultimate goal in the bioremediation of organic compounds contaminating the environment is to develop a population of organisms that are able to utilize the targeted compounds as carbon sources for growth as they convert the compounds into carbon dioxide and biomass. Although the *Rhodococcus* isolates in this study have a narrow substrate specificicty towards PAHs as growth substrates, limited to anthracene, they have a cytochrome P450-like monooxygenase enzyme which has a broad substrate specificity. As shown with *Rhodococcus* strain S1, this bacterium is able to oxidize various kinds of PAHs by cometabolism to transformation products which accumulate and are not metabolized further by this bacterium. The transformed products can be more or less harmful than the parent compounds. In pure culture, cometabolism is a dead-end transformation without benefit to the organism but oxidation leads to an increase in aqueous solubility and availability to other organisms. In natural environments, such an initial cometabolic transformation may prepare the compounds for subsequent attack by other organisms which eventually may lead to recycling of recalcitrant compounds that do not support the growth of any selected pure microbial culture.

6.2. To enhance bioavailability of PAHs.

Low water solubility of PAHs is one factor that limits their availability to microorganisms which in turn affects bioremediation of PAHs. Surfactants may enhance the bioavailability of hydrophobic compounds but both negative and positive effects of surfactants on PAH removal have been reported (Laha and Luthy 1991, Tiehm 1994, Liu *et al.* 1995, Providenti *et al.* 1995, Volkering *et al.* 1995, Soeder *et al.* 1996).

The important aspect of adding surfactants is to stimulate biodegradation when the process is limited by dissolution rate. Adding surfactants to PAH-contaminated sites does not always guarantee enhancement of biodegradation because the interaction among types of surfactants, concentrations, types of PAH, and strains of microorganisms is complicated. A potential alternative to the addition of surfactants is the use of biosurfactant-producing and PAH-degrading microorganisms at the contamination sites. The CMN bacterial group including *Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Rhodococcus* normally synthesize "cord factor", a dimycolyl ester of trehalose, which is claimed to be an extremely powerful emulsifying agent (Barkesdale and Kim 1977, Cooper and Zajic 1980). Such an emulsifying property would increase the availability of hydrophobic contaminants not only to the producing organisms themselves but also to those others which reside in the same habitat. This would reduce the problems that may occur when synthetic surfactants are added such as the microorganisms using the added surfactants rather than PAHs as carbon sources for growth, that the surfactants are toxic to microorganisms, that surfactants themselves contaminate the environment, and the cost of surfactants. There was indirect evidence that *Rhodococcus* S1 produced a surfactant to disperse the anthracene crystals in the growth medium.

In addition, the cell wall of CMN bacteria contain mycolic acids which are α-branched β-hydroxylated long chain fatty acids. These mycolic acids make the cell surface extremely hydrophobic, which permits direct contact with the hydrophobic PAHs and may enhance utilization of the compounds. PAHs are hydrophobic compounds and when they contaminate soil they tend to sorb to soil particles which makes them unavailable for biodegradation. *Rhodococcus* S1 demonstrated an ability to adhere to anthracene as shown by scanning electron microscopy; this would make *Rhodococcus* S1 a good candidate to degrade PAHs that are strongly sorbed to soil.

6.3. Application in chemical and pharmaceutical industries.

Since *Rhodococcus* S1 has the ability to transform several kinds of PAH to various types of oxygenated products, it may have application in the chemical and pharmaceutical industries. It may be used as a microbial biocatalyst to synthesize some compounds that are not readily obtained by conventional chemical syntheses.

7. FUTURE SUDIES.

The *Rhodococcus* sp. strain S1 isolated and used in this study is an unusual bacterium and is different from other anthracene-degrading Gram negative and Gram positive bacteria, including *Rhodococcus* sp. strain UW1 isolated by Walter and colleagues. Of several PAHs tested, it can utilize only anthracene as the sole source of carbon and energy for growth. However, it can cometabolize other PAHs when it grows on anthracene and there is evidence that a cytochrome P450-like monooxygenase enzyme is responsible for this cometabolism. Therefore, in addition to data obtained in this study some future work should be done in order to clarify the pathway this isolate uses to metabolize anthracene for growth and to cometabolize other PAHs.

Recently, there have been several reports from the research group of D.T. Gibson demonstrating varied reactions from a single enzyme, naphthalene dioxygenase, from *Pseudomonas* strains. This enzyme catalyzes various oxidation reactions including *cis*-dihydroxylation of many related 2- and 3-ring aromatic and naphthenoaromatic compounds, monohydroxylation, desaturation (dehydrogenation), *O*- and *N*-dealkylation and sulfoxidation reactions. It would be of interest to examine how *Rhodococcus* S1 metabolizes PAHs for comparison, to see if the naphthalene dioxygenase characteristics are widespread. Some aspects which might be further investigated are described below.

7.1 Metabolic pathway studies.

7.1.1 Anthracene metabolic pathway.

In this study no anthracene metabolites have been detected since they are probably further metabolized without leakage from the cell and do not accumulate in detectable amounts. Thus, it cannot be concluded that *Rhodococcus* S1 does not utilize a pathway

similar to that proposed for Gram negative pseudomonads. However, the anthracene metabolic pathway of *Rhodococcus* S1 has not been yet elucidated. Mutation of *Rhodococcus* S1 by chemical means or transposon mutation to block metabolism at various steps in the pathway would provide an accumulation of intermediate metabolites. Chemical identification of these intermediates would in turn allow a catabolic pathway for anthracene to be proposed.

7.1.2 Confirmation of identity of other PAH metabolites.

Rigorous identification of the tentatively identified transformation products from other PAHs should be completed to achieve a better understanding of how other PAHs are cometabolized. There might be some unidentified metabolites produced, other than those detected in this study, whose detection and identification would contribute to this understanding. Larger scale of metabolite production and radioactively labeled PAHs may be needed to be able to detect trace metabolites.

7.2 Enzymes responsible for PAH transformation.

The initial ring hydroxylating enzyme plays an important role in the bacterial catabolic pathway of PAHs. This enzyme catalyzes the incorporation of two atoms of oxygen into the aromatic ring to form a dihydrodiol in the *cis*-configuration which would be further metabolized and lead to ring cleavage (Gibson and Subramanian 1984).

Cytochrome P450 is also involved in microbial PAH metabolism, mainly by fungi. This enzyme catalyzes the incorporation of only one atom of oxygen into the aromatic ring to eventually yield the corresponding dihydrodiol in the *trans*-configuration. This compound is not metabolized further resulting in accumulation of that transformed product (Cerniglia *et al.* 1992). As described earlier, *Rhodococcus* S1 can utilize only anthracene for growth, presumably by a narrow-specificity dioxygenase, and cometabolize several

PAHs using cytochrome P450-like monooxygenase. It is of interest to investigate the enzymes responsible for these activities in *Rhodococcus* S1.

7.2.1 Anthracene dioxygenase.

The initial enzyme responsible for anthracene metabolism, presumably anthracene dioxygenase, should be investigated because it plays an important role in initial ring oxidation to start the anthracene degradative pathway. Since *Rhodococcus* sp. strain S1 has a narrow PAH-specificity for carbon sources, it may be the substrate specificity of this enzyme which determines the growth spectrum. However, experiments on reconstituted dioxygenase components suggest most dioxygenases have a broad specificity (Furukawa *et al.* 1993). Isolation, purification, and characterization of the dioxygenase enzyme from *Rhodococcus* S1 would be helpful to understanding the properties of the enzyme.

7.2.2 Cytochrome P450 monooxygenase.

Since *Rhodococus* S1 also exhibits cytochrome P450-like monooxygenase activity by oxidizing some non-growth substrate PAHs, the enzyme responsible for these activities should be also investigated.

7.3 Genetics of anthracene metabolism in *Rhodococcus* S1.

The most well studied genes responsible for PAH degradation are the genes for naphthalene degradation. These genes are located on self-transmissible NAH plasmids in those bacteria where they have been studied, mainly in *Pseudomonas* spp. They can be transferred to other strains by conjugation and the genes can be expressed in the recipient strains (Sanseverino *et al.* 1993). Recently there have been reports of pseudomonads harboring NAH7 which are able to mineralize anthracene and phenanthrene in addition to naphthalene. Preliminary studies performed in our laboratory on *Rhodococcus* S1 showed

some evidence that this strain might contain both circular and linear plasmids. There have been reports that enzymes responsible for degradation of biphenyl, polychlorinated biphenyls, and dibenzothiophene are encoded by genes resident on plasmids of *Rhodococcus* spp. (Denome *et al.* 1994, Masai *et al.* 1995, McKay *et al.* 1997). Dabrock *et al.* (1994) reported on a transmissible linear plasmid of *Rhodococcus erythropolis* BD2 which encodes isopropylbenzene and trichloroethane catabolism. Therefore, an investigation of the existence of plasmids in *Rhodococcus* S1 and their involvement in PAH degradation would be a long-term goal.

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APPENDIX.

SOURCES OF CHEMICALS USED.

All chemicals (excluding inorganic salts used for media) used in this study are listed, alphabetically, along with the company from which they were obtained. All ^{14}C-labelled compounds were from Amersham Corp. (Arlington Heights, Illinois). Organic solvents used for extractions, TLC and HPLC analyses, including dichloromethane, ethyl acetate, acetronitile, ethanol, and methanol, as well as acids like HCl, H_2SO_4, and CH_3COOH were obtained from different sources over the years.

Chemicals	Source
Acetic anhydride	Fisher Scientific Co. (Fair Lawn, New Jersey)
Acenaphthene, 97%	Aldrich (Milwaukee, Wisconsin)
ACS Fluor	Amersham
Acetone	BDH (Poole, England)
1-aminoanthracene, tech., 90%	Aldrich Chemical Co. (Milwaukee, Wisconsin)
2-aminoanthracene, 96%	Aldrich
Ancymidol	Sigma Chemical Co. (St. Louis, Missouri)
Anthracene, 99+%	Sigma
Anthraquinone, 99+%	Fluka (Ronkonkoma, New York)
Anthrone, 97%	Aldrich
Benzene	Fisher Scientific Co.
1,2-Benzanthracene, 99%	Aldrich
Biphenyl, 99%	Aldrich
N,O-Bis(trimethylsilyl)acetamide)	Pierce Chemical Co. (Rockford, Illinois)
9-Bromoanthracene, 94%	Aldrich
Bromcresol green	Aldrich
1-Butaneboronic acid, 97%	Aldrich
Carbo-Sorb II	Packard Instruments (Downer's Grove, Illinois)

Catechol, 99+%	Aldrich
1-Chloroanthracene, 97%	Aldrich
2-Chloroanthracene, 95%	Aldrich
9-Chloroanthracene, tech., 75%	Aldrich
Chloroform	BDH
Chloroform (deuterated)	Cambridge Isotope Laboratories (Andover, Maryland)
Chrysene, 98%	Aldrich
Cupric nitrate trihydrate	Aldrich
Cuprous oxide, 97%	Aldrich
o-Dianisidine, 97%	Aldrich
Dibenzothiophene	Fluka
Diethyl ether	BDH
2,2'-Dihydroxybiphenyl, 99%	Aldrich
2,3-Dihydroxybiphenyl	Wako Pure Chemicals (Osaka, Japan)
4,4'-Dihydroxybiphenyl, 99+%	Aldrich
1,2-Dihydroxynaphthalene, tech.	Aldrich
1,3-Dihydroxynaphthalene, 99%	Aldrich
1,5-Dihydroxynaphthalene, 97%	Aldrich
1,6-Dihydroxynaphthalene, 99%	Aldrich
2,3-Dihydroxynaphthalene, 98%	Aldrich
2,6-Dihydroxynaphthalene, 98%	Aldrich
2,7-Dihydroxynaphthalene, 97%	Sigma
N,N'-Dimethylformamide	Sigma
Dimethylsulfoxide	Caledon Laboratories Ltd. (Toronto, Ontario)
EDTA	Aldrich
Fluorene, 98%	Aldrich
Fluoranthene, 98%	Aldrich
2-Fluorenol, 99%	Aldrich
9-Fluorenol, 96%	Aldrich
Formaldehyde	Anachemia (Montreal, Quebec)
Gibb's reagent	Sigma
Glucose	BDH Inc. (Toronto, Ontario)
Glutaraldehyde	Sigma
2-Hydroxybiphenyl, 99+%	Aldrich

3-Hydroxybiphenyl, 90%	Aldrich
4-Hydroxybiphenyl, 97%	Aldrich
1-Hydroxy-2-naphthoic acid, 99%	Aldrich
2-Hydroxy-3-naphthoic acid, 98%	Aldrich
Isopentanol	Anachemia
Kanamycin	Sigma
Light petroleum ether, b.p. 60-80°C	BDH
Lithium aluminium hydride, 95%	Aldrich
Lysozyme	Sigma
1-Methylanthracene, 99%	Aldrich
2-Methylanthracene, 97%	Aldrich
9-Methylantracene, 98%	Aldrich
Methylene chloride (deuterated)	Cambridge Isotope Laboratories
1-Methyl-3-nitro-1-nitrosoguanidine	Aldrich
2-Methylphenanthrene, 95%	Aldrich
Metyrapone	Sigma
Naphtalene, 99+%	Sigma
Naphthacene, 98%	Aldrich
1-Naphthol, 99+%	Aldrich
2-Naphthol, 99%	Aldrich
1,2-Naphthoquinone, 97%	Aldrich
1,4-Naphthoquinone, 95%	Aldrich
Osmium tetroxide	Sigma
Phenanthrene, 99.5+%	Aldrich
Phenanthrene-9,10-quinone, 99+%	Aldrich
9-Phenanthrol, tech.	Aldrich
Phenol (liquefied), certified	Fisher
Phenylboronic acid, 97%	Aldrich
o-Phenylenediamine	Sigma
Phosphomolybdic agid	Sigma
o-Phthalic acid, 98%	Aldrich
Poly-L-lysine hydrochloride	Sigma
Protocatechuic acid, 97%	Aldrich
Pyrene, 99%	Aldrich
RNase	Sigma

Salicylaldehyde, 98%	Aldrich
Salicylic acid, 99+%	Aldrich
Silica gel	General Intermediates of Canada (Edmonton, Alberta)
Sodium bicarbonate	Fisher
Sodium carbonate	BDH
Sodium dodecyl sulfate	Boehringer
Sodium nitrite	Fisher
Sodium perchlorate	BDH
Trichloroacetic acid	Mallinckrodt (Montreal, Quebec)
Toluene	Mallinckrodt
Urea	Fisher